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(54) Title: EQUINE HERPESVIRUS-1 VACCINE

(57) Abstract

Recombinant pox viruses which express antigens of equine herpesvirus-1 are disclosed. The recombinant pox virus can be used to vaccinate horses and other animals against equine herpesvirus-1 infection.

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EQUINE HERPESVIRUS-1 VACCINE

Background of the Invention

Viruses classified as Equine Herpesvirus Type 1 (EHV-1) are a major cause of abortion and respiratory disease in horse populations throughout the world. They are also associated with neonatal foal disease and a neurological disease with paralysis. Together these diseases represent a significant financial loss to the equine industry each year.

There are two distinct subtypes of EHV-1 that differ in antigenicity, tissue culture characteristics, epizootiology, pathogenicity, and molecular biology. Both subtypes cause respiratory disease in young horses, and both have potential to cause abortion; however, only subtype 1 has been associated with abortion "storms" and the neurological syndrome.

The immune response to EHV-1 infection is poorly understood. Once maternal antibodies have waned, the immune response elicited by respiratory infection with EHV-1 is short lived and horses may be reinfected every 3-4 months. Consecutive infections however, do, appear to induce some measure of protection, as clinical signs of disease are seldom seen in the older animal. The immune response elicited by abortion is more protective than that stimulated by EHV-1 respiratory disease and the majority of mares do not abort as a result of EHV-1 infection more than once. Nevertheless, mares with high levels of specific virus neutralizing antibody are susceptible to reinfection

with EHV-1, which suggests that cell-mediated immunity may play an important role in protection.

EHV-1 vaccines currently available consist of live, attenuated or killed EHV-1 subtype 1. An inactivated, adjuvanted EHV-1 vaccine is of limited efficacy against either subtype of EHV-1. The live attenuated vaccine is claimed to protect against respiratory disease, but cannot, according to the manufacturers, guarantee protection against abortion. Furthermore, live vaccines have been associated with anaphylactoid reactions, abortion, or neurological damage. In molecular epizootiological surveys in Kentucky, a number of viruses isolated from aborted fetuses had the distinctive electrophorotype of vaccine strains.

A need therefore exists for a safe and effective vaccine for EHV-1. Recombinant DNA techniques offer new approaches for the development of such a vaccine. These methodologies make possible the use of defined antigens, rather than the intact infectious agent, as immunogens. These include peptide vaccines, consisting of chemically synthesized, immunoreactive epitopes; subunit vaccines, produced by expressing viral proteins in recombinant heterologous cells; and the use of live, recombinant viral vaccines for the presentation of one or more devined antigens.

Both peptide and subunit vaccines are subject to a number of potential limitations. First, it is difficult to ensure that the conformations of the engineered proteins mimic those of the entigens in their natural environment. If the vaccine structure

differs from the natural protein structure, the vaccine might not be protective. Second, even if the antigens are properly presented, suitable adjuvants and, in the case of peptides, carrier proteins must be used to boost the immune response. Finally, peptide and subunit vaccines elicit primarily humoral responses and thus may fail to provide adequate protection. Many responses and thus may fail to provide adequate protecton. Many of the problems associated with the use of peptides and subunit vaccines can be overcome through the use of live recombinant viral vaccines.

Vaccinia virus has been used in the worldwide eradication of smallpox. Its effectiveness in the vaccination program is due to its relative safety, stability, ease of administration and low cost. Vaccinia virus, a DNA virus, has several advantageous characteristics for use as a vector for creating live recombinant vaccines: it permits relatively easy genetic manipulation; it has a genome which can accept a large amount of foreign DNA; it is not oncogenic, are easy to grow and purify, and they have an extremely wide host range, infecting both man and animals.

Paoletti et al. (U.S. Patent No. 4,603,112) have developed a technique known as <u>in vivo</u> recombination for integration of foreign DNA into vaccinia virus. Several foreign genes can be recombined into one virus using this technique, but each gene must be inserted individually.

A means of utilizing vaccinia virus as a eukaryotic vector has recently been developed. It has been demonstrated that foreign DNA sequences can be inserted into the genome or vaccinia virus by a process of site specific homologous recombination between replicating vaccinia genomes and appropriate vaccinia DNA sequences which flank the foreign DNA of interest (Panicali et al., Proc. Natl. Acad. Sci. USA. 1982. Vol. 79, pgs 4927-4931). Recombinant viruses have been created in this manner to contain and express DNA sequences which code for proteins of pathogenic organisms. These recombinant viruses can serve as vaccines for protection against infection with the corresponding pathogen.

Summary of the Invention

This invention pertains to recombinant pox viruses capable of expressing immunogenic proteins and glycoproteins of EHV-1 and to the use of these recombinant viruses for vaccination against EHV-1 and for the production of equine herpesviral antigens. The invention also pertains to DNA vectors containing EHV-1 DNA sequences for recombination with pox virus to produce recombinant pox viruses which express protein(s) encoded by the inserted DNA sequences. Di- and multivalent vectors allow two or more EHV-1 genes to be inserted together by a single in vivo recombination event. The vectors can be used to create monovalent and multivalent pox viruses containing and expressing the genes encoding immunogenic proteins or glycoproteins of EHV-1 such as

the envelope glycoproteins gB and gH. The recombinant pox viruses can be used to induce humoral and cell mediated immunity to EHV-1 in horses and other animals susceptible to virus infection.

Brief Description of the Figures

Figure 1 shows the BamHI and BgIII restriction endonuclease cleavage maps of the genome of EHV-1 subtype 1.

Figure 2 shows the structure of three plasmids that contain cloned restriction fragments from the EHV-1 genome. Figure 2a shows the structure of pAbT775, which contains the BamHI i fragment cloned in the plasmid vector pEMBL18+; Figure 2b shows the shows the structure of pAbT354, which contains the BamHI b fragment cloned in the plasmid vector pEMBL18+; and Figure 2c shows the structure of pAbT791, which contains the BgIII g fragment cloned into the plasmid vector pUC13.

Figure 3 shows the DNA sequence of approximately 80% of the EHV-1 gB coding sequence, including the initiation and termination codons of the structural gene as well as 5' and 3' non-coding sequences.

Figure 4 shows the construction of plasmid pAbT817, a vector for the insertion of the EHV-1 gB gene into vaccinia virus with the gB gene under the control of the vaccinia 40K promoter.

Figure 5 shows the construction of plasmid pAbT 813, a vector for the insertion of the EHV-1 gH gene into vaccinia, with the gH gene under the control of the vaccinia 7.5K promoter.

Figure 6 shows the construction of pAbT4587, a plasmid vector for insertion of foreign genes into vaccinia by in vivo recombination (IVR) containing the vaccinia 40K promoter, the vaccinia HindIII M region for direction of recombination, the 20K host range gene for selection of recombinants, and a bacterial replicon and ampicillin resistance gene for propagation in E.coli.

Figure 7 shows the construction of plasmid pAbT829, a vector for the insertion of the EHV-1 gH gene into vaccinia, with the gH gene under the control of the vaccinia 40K promoter.

Figure 8 shows the construction of plasmid pAbT822, a vector for the simultaneous insertion of the EHV-1 gB and gH genes into vaccinia virus, with the gB gene under the control of the 40K promoter and and the gH gene under the control of the 7.5K promoter.

Figure 9 shows immunoprecipitation analysis of recombinants vAbT243 and vAbT249. Figure 9a shows the results of immunoprecipitation of [^{35}S]-methionine labeled cells infected with vAbT243 (lanes 2, 4 and 6) or the wild type NYCBH vaccinia strain (lanes 1, 3 and 5). The antibodies used were rabbit anti-EHV-1, purchased from the American Type Culture Collection (ATCC), cat. #VR-700 AS/Rab (lanes 1 and 2); goat anti-PRV gII (lanes 3 and 4); rabbit anti-EHV-1, generated at Applied bio Technology, Cambridge, MA (lanes 5 and 6). The positions of the molecular weight markers are indicated. Fig. 9b shows the results of immunoprecipitation of [^{3}H]-glucosamine

labeled cells infected with vAbT243 (lane 2) or the wild type NYCBH vaccinia strain (lane 1). The antibody used was rabbit anti-EHV-1 purchased from ATCC. Figure 9c shows the results of immunoprecipitation of [^{35}S]-methionine labeled cells infected with vAbT249 (lane 2) or with the wild type NYCBH vaccinia strain (lane 1). The antibody used was rabbit anti-EHV-1 purchased from ATCC.

Figure 10 shows the results of immunoprecipitation of [^{35}S]-methionine labeled cells infected with EHV-1. The antibodies used were: lane 1, normal mouse serum; lane 2, mouse anti-vaccinia; lane 3, mouse anti-vAbT243; lane 4, mouse anti-vAbT249; lane 5, mouse anti-vAbT244. The positions of the molecular weight markers are indicated.

Figure 11 shows the results of EHV-1 plaque-reduction assays performed using mouse antisera generated against vaccinia recombinants vAbT243, vAbT244, and vAbT249.

Detailed Disclosure of the Invention

1. EHV-1 Genes for integration into pox virus

EHV-1 genes for integration into the genome of a pox virus in expressible form can be obtained by any conventional technique for isolating a desired gene. For purposes of a vaccine, genes of interest are those which encode immunogenic proteins of EHV-1. In many cases, these are protein components of the viral envelope. Immunogenic fragments or subunits of the proteins may be used.

Eight major glycoproteins have been identified in the envelope of EHV-1. The major glycoproteins, which correspond to EHV-1 structural proteins have been designated VP2, 10, 13, 14, 17, 18, 21, and 22a with respective molecular masses of 200, 125, 95, 90, 68, 63, 45, and 41 kilodaltons (M.L. Perdue, et al., 1974. Virology 59, 201; L.W. Turtinen and G.P. Allen, 1982. J. Gen. Virology 63,481).

A comparison of the EHV-1 genome to that of the well-characterized herpes simplex virus-1 HSV-1 genome by DNA hybridization studies suggested that the two viruses share a similar genetic organization. Both viruses contain a long unique sequence (U_L) contiguous with a short unique sequence (U_S) which is flanked by inverted repeats. Regions of homology in the two genomes detectable by DNA hybridization are arranged colinearly in the U_L. This similarity made it possible to predict the approximate locations of a number of EHV-1 glycoprotein genes encoded in the U_L region based on the genomic locations of their HSV-1 counterparts. In addition, restriction endonuclease linkage maps of the EHV-1 genome for the enzymes BamHI, EcoRI, and BglII have been published (Whalley et al., 1981. J. Gen. Viro 57:307).

One of the glycoprotein genes encoded in the U_L, designated gB in HSV-1 and vp14 in EHV-1, encodes an essential glycoprotein which plays an important role in virus penetration and stimulates both humoral and cell mediated immunity. The extent of structural conservation of this protein in the Herpesviridae

indicates that its essential function is likely to be conserved.

A second glycoprotein encoded in the HSV-1 U_L region, designated gH, is essential for efficient release of virus from the infected cell and appears to be a major target for neutralizing antibody.

For EHV-1, the genes encoding the antigen of interest are isolated form the genomic DMA. Isolation of these genes requires a knowledge of their genomic location, which may have been proviously determined or which can be determined by a number of conventional techniques. The available restriction maps of EHV-1 make possible the design of strategies for cleaning genomic DNA by restriction endonuclease digestion, to yield DNA fragments that contain the gene of interest. Ultimately, DNA sequence analysis must be used to locate precisly the DNA sequence that encodes the antigen of interest. The DNA sequence analysis may also reveal convenient restriction endonuclease cleavage sites that can be used to clone the gene into appropriate plasmid vectors. These genes can be amplified by cloning the gene into a bacterial host. For this purpose, various prokaryotic cloning vectors can be used. Examples are plasmids pBR322 and pEMBL.

The genes encoding the antigen of interest can be prepared for insertion into the DNA vectors designed for recombination with pox virus by standard techniques. In general, the cloned genes can be excised from the prokaryotic cloning vector by restriction enzyme digestion. In some cases, the excised fragment will contain the entire encoding region of the gene,

including its translational start signal; in others, the translational start signal will be absent. The DNA fragment carrying the cloned gene can be modified as needed, for example, to make the ends of the fragment compatible with the insertion sites of the DNA vectors used for recombination with poxvirus, then purified prior to insertion into these vectors at restrictuion endonuclease cleavage sites (cloning sites) as described below.

As described in more detail below, encoding EHV-1 glycoproteins homologous to HSV-1 gB and gH were isolated from the genomic DNA of and cloned into E. coli plasmid vectors. After amplification, the protein-encoding region of each gene, including its translational start signal, was excised from the bacterial plasmid by restriction endonuclease cleavage and inserted into the pox recombination vectors. Other examples of EHV-1 virus glycoproteins which can be inserted into pox virus using the same tecnnique include VP 2, 10, 13, 17/18, and 21/22a.

2. Pox viruses

Any member of the pox family can be used for the generation of recombinant viruses expressing EHV-1 antigens. For the purposes of vaccine development, the preferred pox virus is a virus which does not cause significant disease in horses and other animals. The most preferred pox virus is vaccinia virus, a relatively benign virus, which has been used for years as a vaccine against smallpox in humans. Several strains of vaccinia, which differ in level of

virulence, are available for use as vaccine strains; for the purposes of vaccination, a less virulent strain such as the New York State Board of Health Strain which still retains the ability to elicit an appropriate immuune response is preferred. General techniques for integration of foreign DNA into vaccinia virus to produce a modified virus capable of expressing foreign protein encoded by the foreign DNA are described by Paoletti et al U.S. Patent No. 4,603,112, the teachings of which are incorporated by reference herein.

3. DNA vectors for recombination with pox virus

According to the method of this invention, DNA sequences which encode immunogenic proteins of EHV-1 are inserted into the genome of a pox virus to create a recombinant pox virus which expresses the EHV-1 protein along with the expression of the normal complement of pox virus proteins (except for the pox viral protein encoded by a gene into which the foreign DNA is inserted). This is accomplished by first constructing a DNA donor vector for in vivo recombination with pox virus.

In general, the donor vector contains (i) a prokaryotic origin of replication so that the vector may be amplified in a prokaryotic host, (ii) a gene encoding a marker which allows selection of prokaryotic host cells that contain the vector (e.g., a gene conferring antibiotic resistance) (iii) DNA sequences homologous to the region of the pox virus genome where EHV-1 gene is to be inserted, (iv) one or

more EHV-1 genes inserted at a site within this pox virus sequence and (v) one or more pox virus regulatory sequences (promoters) adjacent to the 5' end of each EHV-1 gene, constructed in a manner to allow for transcription of the EHV-1 gene and subsequent expression.

A method for constructing expression vectors for the introduction of single or multiple foreign genes into pox virus is described in co-pending U.S. patent application Serial No.910,501, filed September 23, 1986, entitled "Pseudorabies Vaccine", the techniques of which are incorporated herein by reference. In general, all pox virus DNA fragments for construction of the donor vector, including fragments containing transcriptional promoters and fragments containing sequences homologous to the region of the pox virus genome into which EHV-1 DNA is to be inserted, can be obtained from genomic DNA or cloned DNA fragments. The vectors can be mono, di or multivalent (i.e. have one or more inserted EHV-1 gene sequences). In the di- or multivalent vectors, each EHV-1 gene should be placed under control of a different pox viral promoter. The vector backbone can be derived from any of several plasmid vectors capable of replication in a prokaryotic host, e.g., pBR322 (Sutcliffe, Cold Spring Harbor Symp., Quant. Biol., 43:77 (1979), pUC8 (Vieira and Messing, Gene, 19:259 (1982) or pEMBL (Denta et al., Nucleic Acids Res., 11:1645 (1982)). The ability to replicate in a prokaryotic host provides a means for amplification of the vector to produce sufficient

quantities for transfection of a eukaryotic cell for recombination.

The donor vector preferably contains an additional gene which encodes a marker which will allow selection of recombinant pox viruses containing integrated EHV-1 DNA. The gene encoding the marker is placed under control of a pox virus promoter. Several types of marker genes can be used. A preferred marker gene is the E. coli lacZ gene which encodes the enzyme beta-galactosidase. Recombinant pox virus will express beta-galactosidase along with the EHV-1 antigen. Thus, beta-galactosidase production is detected as an indication of insertion and expression of the EHV-1 DNA. In the presence of a chromogenic indicator (BluoGal) recombinant viruses which express beta-galactosidase will form blue plaques. Furthermore, a recombinant virus that has sequences inserted into the pox virus TK (thymidine kinase) gene will have an inactivated TK gene and will grow in the presence of BUdR, while virus containing a wild-type, uninterrupted TK gene will be unable to grow in the presence of BUdR (Dubbs and Kit (1964) Virol., 22:214; Smith et al. (1983) Proc. Natl. Acad. Sci., USA, 80:7155). Other selectable markers include genes which confer antibiotic resistance in an infected host cell, e.g., the Neo^R gene. Infected cells are grown in media containing the antibiotic at a concentration toxic for antibiotic sensitive cells. Under these conditions, cells infected with a recombinant virus expressing the resistance marker will produce virus.

Cells infected with virus not containing the marker will not produce virus.

Another procedure for selection of recombinant viruses relies upon a single vaccinia-encoded function. This procedure obviates the need for the use of drugs, mutagens, or chromogenic indicators, and permits rapid (2-3 wk) purification of recombinant viruses. The method is described in co-pending U.S. patent application Serial No. 205,189, filed June 20, 1988, entitled "Method of Selecting For Recombinant Pox Viruses", the teachings of which are incorporated herein by reference. Briefly, a vaccinia virus is employed that contains a mutation in a specific structural gene (29K gene), located in the HindIIIM fragment of vaccinia virus. This mutation prevents the growth of the virus on particular host cell, for example RK13 (rabbit kidney) cells. The intermediate DNA vector contains vaccinia DNA sequences capable of restoring the mutant gene function; these sequences also direct recombination to the site of the mutant gene in the HindIIIM region. Thus, recombinant vaccinia viruses regain the ability to grow on RK13 cells, and can be isolated from non-recombinant viruses, which are unable to grow on these cells.

A preferred DNA vector for recombination with the preferred vaccinia virus comprises:

a. one or more vaccinia promoters (e.g., the vaccinia 11K, 7.5K, 30K, 40K or BamF promoter or modified versions of these promoters), each linked to;

b. one or more structural genes encoding EHV-1 antigens of interest (e.g., the gB, gH antigen) each under control of a promoter;

c. a marker for the selection of recombinant vaccinia virus, which may comprise:

(1) a vaccinia promoter (e.g., the BamF promoter of vaccinia virus) linked to a gene encoding a selectable marker (e.g., the E. coli lacZ gene); or

(2) vaccinia structural gene sequences which restore the function of the 29k polypeptide;

d. DNA sequences homologous with a region of vaccinia nonessential for replication flanking the construct of elements a-d (e.g., the vaccinia TK or HindIIM sequence).

e. a vector backbone for replication in a prokaryotic host including a marker for selection of bacterial cells transformed with the plasmid (e.g. antibiotic resistance).

4. In vivo recombination

The intermediate DNA vectors containing the EHV-1 gene(s) and the marker gene flanked by appropriate pox viral sequences undergo recombination with pox virus genomic DNA, which results in integration of the flanked gene into the viral genome. Recombination occurs in a eukaryotic host cell. Appropriate host cells for recombination are those which are 1) infectable by pox virus and 2) transfectable by the DNA

vector. Examples of such cells are chick embryo fibroblast, CV-1 (monkey kidney cells), HuTk 143 cells (human cells), BSC40 (monkey kidney cells), RK-13 cells (rabbit kidney cells) and many others

Viral infection is accomplished by standard techniques for infection of eukaryotic cells with pox virus. See e.g., U.S. Patent No. 4,603,112.

The cells can be transfected with the intermediate vector by any of the conventional techniques of transfection. These include the technique of calcium phosphate precipitation, DEAE dextran, electroroporation and protoplast fusion. The preferred technique is the calcium phosphate precipitation technique.

After infection and subsequent transfection, the cells are incubated under standard conditions and virus is allowed to replicate, during which time in vivo recombination occurs between the homologous pox virus sequences in the intermediate vector and the pox virus sequences in the genome.

Recombinant viral progeny are then identified by any of several techniques. For example, virus harboring the EHV-1 gene(s) can be selected on the basis of inactivation of the viral gene into which the EHV-1 foreign DNA was inserted. For example, if the DNA vector is designed for insertion into the TK gene, viruses containing integrated DNA will be TK^- and can be selected on this basis. Preferred techniques for selection, however, are based upon co-integration of a gene encoding a marker or indicator gene as described above. One preferred indicator gene is the E. coli

lacZ gene. Selection of recombinant viruses expressing beta-galactosidase can be done by employing a chromogenic substrate for the enzyme. A second preferred indicator gene is the vaccinia 29K gene. Selection of recombinant viruses that express the wild-type 29K gene-encoded function can be performed by growing the recombinant virus on RK-13 cells.

As described more fully below, monovalent and divalent donor vectors containing the genes encoding EHV-1 glycoproteins gH and/or gB were recombined into vaccinia at the TK gene and recombinants were selected as blue plaques using the lacZ gene and BluoGal™ substrate. Alternatively, the gH gene was recombined into vaccinia at the Hind III M sequence and selected by growth on RK-13 cells due to expression of the wild type 29K gene-encoded functions. Expression of the protein encoding these equine herpes virus glycoproteins in the vaccinia recombinants was confirmed by various immunological methods.

5. Vaccines

Live recombinant viruses expressing immunogenic proteins of EHV-1 can be used to vaccinate horses and other animals susceptible EHV-1 infection. These vaccines may be administered intradermally, or by other routes such as intramuscular, subcutaneous, and oral routes. Vaccination of a horse with live recombinant vaccinia virus is followed by replication of the virus within the host. During replication, the gene is expressed along with the normal complement of vaccinia genes. The EHV-1 gene product will stimulate

the host to mount an immunological response, both humoral and cell mediated, to the foreign antigen as well as to vaccinia virus itself.

Live recombinant vaccinia viruses containing and expressing one or more of the genes encoding equine glycoproteins such as gB, gH or other immunogenic proteins provide monovalent, divalent and multivalent vaccines for immunizing horses and other animals against equine rhinopneumonitis, abortion, neonatal foal disease and a neurological disease with paralysis caused by EHV-1.

An additional advantage of utilizing recombinant pox viruses as live vaccines against EHV-1 is that they express only selected antigens, preferably only those antigens sufficient to elicit a protective response. It is therefore possible to differentiate between host animals which have been vaccinated with the recombinant pox virus and those which have been infected with the authentic, virulent, disease-causing agent.

The vaccinated host will develop antibodies only to the pox virus and to the selected foreign antigen(s). By contrast, the actively infected host will generate a full complement of antibodies directed toward the pathogenic agent, including antibodies directed to specific antigens not present in the recombinant pox virus. The presence of these additional antibodies, which can be detected using appropriate immunological tests (e.g., ELISA), is therefore diagnostic of a naturally occurring infection of the host with the pathogen.

Using this invention, recombinant vaccinia viruses containing selected EHV-1 antigens can be used as vaccines. Because vaccinated animals will produce antibodies against only those equine herpes viral antigens expressed by the recombinant vaccinia virus used for vaccination, they can be distinguished from animals infected with EHV-1; the infected animals, in contrast to vaccinated animals, will contain antibodies to the entire complement of EHV-1 antigens.

Recombinant pox viruses expressing EHV-1 antigens can also be used to generate immunogenic proteins in culture which can be used as subunit vaccines. Antigens for subunit vaccine preparations can be produced by infecting eukaryotic cell cultures with one or more recombinant viruses, each expressing one or more foreign antigens. The infected cell will express these foreign antigens along with the complement of pox virus proteins. These antigens can then be utilized as a subunit (non-live) vaccine preparation. The EHV-1 antigens may be purified from the infected cells for administration or they may be given as a crude lysate of infected cells. Under these circumstances, any live recombinant pox viruses present would have to be inactivated, which can be done by conventional techniques such as the use of formalin, heat, and ultra violet radiation, among others.

For example, subunit vaccines can be made from the monovalent and divalent recombinant vaccinia viruses containing the genes encoding EHV-1 glycoproteins gB, gH or other EHV-1 glycoproteins.

6. Diagnostic Uses of Recombinant Pox Viruses

Recombinant pox virus which express one or more EHV-1 antigens can also provide diagnostic tools for detection of EHV-1. Infection of experimental animals with the recombinant pox viruses can be used to raise both monoclonal antibodies and polyclonal antisera which recognize the specific epitopes of the EHV-1 protein. These monoclonal and/or polyclonal antibodies can be used individually or together as capture antibody for immunoassay in the RIA or ELISA format, to detect the presence in a biological fluid (e.g., urine, blood, or feces) of EHV-1.

Alternatively, cells infected in vitro with the recombinant pox viruses can be used as a source of the EHV-1 antigen produced by the recombinant viruses for an immunoassay to detect the presence of anti-EHV-1 antibody in urine, blood, or feces of an animal. Particularly preferred immunoassays are solid phase immunometric assays (enzymetric or radiometric). In such assays, the EHV-1 antigen of interest is immobilized on a solid phase to provide an immunoadsorbent. The immunoadsorbent is then incubated with a sample of bodily fluid to be tested under conditions sufficient for antibody reactive with the antigen to complex with ummobilized antigen. The immunoadsorbent is separated from the sample and antibody associated with the immunoadsorbent is determined as an indication of the level in the sample of antibody against the organism which normally contains the antigen. Antibody bound to the immunoadsorbent is generally determined by incubating the immunoadsorbent with a labeled

(radioisotopically or enzymatically) antibody against antibody of the species from which the sample is derived and detecting label associated with the immunoadsorbent.

For example, in the case of recombinant vaccinia viruses expressing EHV-1 glycoproteins, these viruses can be used to infect experimental animals to raise monoclonal antibodies and/or polyclonal antisera which recognize one or more of these glycoproteins. These monoclonal and/or polyclonal antibodies can then be used as capture antibody in an immunoassay for the detection of EHV-1 specific antigens in the urine, blood, or feces of a host animal.

Alternatively, EHV-1 glycoproteins can be isolated in crude or pure form from cells infected in vitro with recombinant vaccinia viruses expressing one or more of these glycoproteins. These glycoproteins can then be used in an immunoassay to detect the presence of anti-EHV-1 antibodies in the ebodily fluid of host animals as described above.

The invention is illustrated further by the following Examples:

EXAMPLES

Cells and Virus

E. coli strain MC1061 (Casadaban and Cohen, 1980, J. Mol. Biol. 138:179) was used as the host for the growth of all plasmids. The monkey kidney cell line BSC-40 (Brockman and Nathans, 1974. Proc Natl Acad Sci USA 71:942), the rabbit kidney cell line RK13 (ATCC

#CCL37; Beale et al., 1963, Lancet, 2:640) and the thymidine kinase-deficient (TK^-) human cell line $Hu143TK^-$ (Bacchetti and Graham, 1977. Proc. Natl. Acad. Sci. USA 74:1590) were used for vaccinia infections and transfections. RK13 cells were also used for the propagation of EHV-1.

Vaccinia virus strain NYCBH (ATCC #VR-325) and 29K- lacZ+ strain vAbT33 (see U. S. Patent Application Serial No. 205,189, filed June 10, 1988, incorporated herein by reference) were used as the parental virus for in vivo recombination.

EHV-1 subtype a strain Kentucky D was obtained from ATCC (#VR-700).

Enzymes

Restriction enzymes were obtained from New England BioLabs or Boehringer-Mannheim. The large fragment of DNA polymerase (Klenow) was obtained from United States Biochemical Corp., and T4 DNA ligase was obtained from Boehringer-Mannheim.

Molecular Cloning Procedures

Restriction enzyme digestions, purification of DNA fragments and plasmids, treatment of DNA with Klenow, T4 DNA polymerase, ligase, or linkers and transformation of E. coli were performed essentially as described (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982).

DNA Sequence Analysis

All DNA sequence analysis was performed using the chain-termination method (Sanger et al., 1977, Proc. Natl. Acad. Sci. USA, 74:5463).

Preparation of EHV-1 DNA

RK13 cells were infected with EHV-1 at a multiplicity of 0.1 in Minimum Essential Medium (Gibco) supplemented with 10% fetal calf serum. DNA was extracted from cell-released or cell-associated virus according to the method of Wilkie for herpes simplex virus (Wilkie, 1973, J. Gen. Virol. 21:453).

Preparation of Vaccinia Virus Recombinants

Viral infection, transfections, plaque purification and virus amplication were performed essentially as described (Spyropoulos et al., 1988, J. Virol., 62:1046). TK- recombinant plaques were selected and purified in the presence of 50 uM bromodeoxyuridine. 29K+ recombinants were selected and purified on RK13 (see U. S. Patent Application Serial No. 205, 189, filed June 10, 1988, incorporated by reference herein).

Vaccinia Virus Genomic Analysis

DNA was extracted from vaccinia virus-infected cells as described (Esposito et al., J. Virol. Methods, 2:175) and analyzed by restriction enzyme digestions and Southern hybridization as described (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982).

Generation of Rabbit Antiserum to EHV-1

Production of EHV-1 virions for generation of EHV-1 antisera was carried out by infecting fourteen

15cm plates of RK13 cells at an MOI of .003 for 1 hour in serum free media, then harvesting cells after sixty hours. Cells and supernatant were banded on a dextran gradient and resuspended in 250 microliters of PBS. This virus stock was then emulsified with an equal volume of complete Freunds adjuvant. One rabbit was injected both subcutaneously and intramuscularly on day zero. First boost on day forty-four consisted of 50, 15cm plates of EHV-1 infected cells in complete Freunds adjuvant. Second boost on day 79 consisted of 10, 15cm plates of EHV-1 infected cells in complete Fruends adjuvant. The rabbit was exsanguinated on day 120.

Protein Analysis

Black plaque assay and immunoprecipation analysis were performed essentially as described in U. S. Patent Application Serial No. 910,501 filed September 23, 1986 (see also European Patent Application No. 87 308370.1). For black plaque assay polyclonal antiserum against total EHV-1 was used. For immunoprecipation analysis, vaccinia-infected cells were labeled with either [^{3}H]-glucosamine or [^{35}S]-methionine, and the following antisera were used: 1) rabbit anti-EHV-1, purchased from ATCC (#VR-700 AS/Rab); 2) rabbitanti-EHV-1, generated using whole EHV-1 virus as immunogen; and 3) goat anti-Pseudorabies virus (PrV) gII, received from Lynn Enquist, E. I. Dupont de Nemours and Company, Wilmington, DE.

Mouse Immunizations

Female Balb/c mice were immunized I.P. with 10^7-10^8 pfu vaccinia virus recombinant. Sera were obtained three and four weeks post-immunization.

Characterization of Mouse Immune Sera

Anti-vaccinia antibody titers of sera from immumized mice were determined by enzyme-linked immunosorbant assay (ELISA), using vaccinia strain WR as antigen, performed essentially as described in U. S. Patent Application Serial No. 910,501, filed Septemer 23, 1986. (See also European Patent Application No. 87 308390.1.)

Immunoprecipitation analysis for characterization of mouse immune sera was carried out essentially as described in U. S. Patent Application Serial No. 910,501, filed September 23, 1986, using EHV-1 labeled with ^{35}[S]-methionine as antigen and mouse immune sera raised to vaccinia/EHV-1 recombinants as antibody. EHV-1 viral proteins were labeled with ^{35}[S]-methionine by infecting RK13 cells at a multiplicity of infection of 3 in 2% fetal calf serum, for one hour at 37°. Harvesting of proteins and lysate preparation were performed as described in U.S. Patent application Serial No. 910,501 filed September 23, 1986. (See also European Patent Application No. 87 308390.1.)

Assessment of neutralizing ability of antibodies raised to vaccinia/EHV-1 recombinants was carried out using a plaque reduction assay. A series of dilutions of mouse immune sera was incubated with a known amount of EHV-1 virus in 2% fetal calf serum/MEM for 1 hour at 37°. RK13 cells were then infected with the above inoculum, for 1 hour at 37°. Inoculum was removed and

0.6% agarose/10% fetal calf serum/MEM overlay was applied and incubated at 37°. Plaque formation was assessed 3 days post infection.

EXAMPLE 1: Cloning Restriction Enzyme Fragments from EHV-1 DNA

Restriction endonuclease linkage maps of the genome of the EHV-1 subtype 1 for the enzymes BamHI, EcoRI, and BglII have been published (Whalley et al., 1981, J. Gen. Virol., 57:307. The BamHI and BglII restriction maps are shown in Fig. 1. EVH-1 DNA, prepared as described in Materials and Methods, was digested with BamHI, and the entire digestion mixture was ligated to the vector pEMBL18+ (Dente et al, 1983, Nucl. Acids Res., 11:1645) which had been digested with BamHI and treated with calf alkaline phosphatase. After transformation of E. coli cells, the resulting plasmids were purified and analyzed by restriction enzyme digestion with BamHI to determine which of the EHV-1 BamHI restriction fragments they contained. Two of the clones, designated pAbT775 and pAbT354, which contain the BamHI i and the BamHI b fragments, respectively, were used for the subsequent experiments. These plasmids are diagrammed in Figs. 2a and 2b.

To clone the BglII g fragment from EHV-1, total EHV-1 DNA was digested with BglII and the BglII g fragment was gel purified and ligated to the plasmid vector pUC13 (Yanisch-Perron et al, 1985, Gene, 33:103) which had been digested with BamHI and treatd

with calf alkaline phosphatase. The resulting plasmid was designated pAbT791 (Fig. 2c).

EXAMPLE 2: Mapping the location of the EHV-1 gene homologous to HSV-1 gB

The EHV-1 gene that corresponds to HSV-1 gB was located on the EHV-1 genome by virtue of of its homology to Herpes Simplex Virus (HSV) gB (Robbins et al., 1985. 10th International Harpesvirus Workshop, Ann Arbor, Michigan). A 6900bp BamHI fragment containing the HSV gB gene in pBR322 was obtained from Dr. Lynn Enquist (E.I. duPont de Nemours and Co., Wilmington, Delaware).

EHV-1 genomic DNA was digested with BamHI or with BglII and the resulting fragments were separated on an agarose gel and transferred to nitrocellulose. The filter was then hybridized to the DNA fragment containing the HSV gB gene, which was labeled with ^{32}P by nick-translation. The BamHI i fragment and the BglII g, x, and b fragments of EHV-1 all hybridized to the HSV gB probe, which localized the coding sequence of the EHV-1 homolog to the left end of the BamHI i fragment. This location has been recently confirmed by the work of Allen and Yeargan (Allen and Yeargan, 1987, J. Virol. 61:2454).

In order to define precisely the boundaries of the EHV-1 gB gene, DNA sequence analysis using the chain-termination method (Sanger et al., 1977, Proc. Natl. Acad. Sci. USA, 74:5463 was performed. Although the entire coding region of the gene was not sequenced, this analysis identified the location of

the initiation and termination codons of the gB structural gene. The nucleotide sequence is presented in Fig. 3.

EXAMPLE 3: Construction of a monovalent IVR vector containing the EHV-1 gene encoding the glycoprotein gB homolog under the control of the vaccinia 40K promoter (Fig. 4)

Because the available restriction sites did not permit cloning of the gB sequences in a single step, a four-part cloning scheme was used. This consisted of the following steps:

Step 1, subcloning a fragment from the EHV-1 BglII g fragment that contains the 5' coding sequence of the gene;

Step 2, joining the 5' sequences to the middle of the gene, which is contained on the BamHI i fragment;

Step 3, attaching the 3' terminus of the gene;

Step 4, inserting the reconstructed gene into an IVR vector.

Step 1: pAbT791, which contains the BglII g fragment of EHV-1 cloned into the BamHI site of pUC13, was digested with SacI and an approximately 800 bp fragment containing the 5' end of the gB gene was gel-purified. This fragment was inserted into the SacI site of the plasmid pEMBL18+ to give plasmid pAbT807.

pAbT807 was digested with EcoRI and HindIII and an approximately 800 bp fragment was gel-purified. This fragment was digested with HgaI, treated with the

large fragment of DNA polymerase (Klenow), then digested with XbaI. An approximately 660 bp fragment resulting from this digest was gel-purified, then ligated into the plasmid vector pEMBL19+ (Dente et al., 1983, Nucl. Acids Res. 11:1645) which had been digested with KpnI, treated with T4 polymerase, and then digested with XbaI. The resulting plasmid was designated pAbT810.

Step 2: pAbT810 was digested with BamHI and HindIII, and an approximately 4400 bp fragment was gel purified. pAbT775, which contains the BamHI i fragment of EHV-1 cloned into the BamHI site of pEMBL18+, was digested with BamHI and HindIII, and an approximately 2200 bp fragment was gel-purified. This fragment was ligated to the BamH1-HindIII fragment of pAbT810 to give the plasmid pAbT812.

Step 3: pAbT812 was digested with HindIII and treated with Klenow. XbaI linkers (New England Biolabs, cat #1032) were ligated to the digested plasmid, and the linkered DNA was then digested with XbaI and KpnI, and a 1250 bp fragment was gel purified. This was ligated to the 5600 bp fragment from pAbT812 to yield pAbT815.

Step 4: pAbT815 was digested with EcoRI and treated with Klenow. XbaI linkers (New England BioLabs, cat #1032) were ligated to the digested DNA; then the DNA was digested with XbaI, and a 2820 bp fragment was gel purified. This was ligated into the XbaI site of pAbT4533 (an IVR vector described in U.S. Patent Application Serial No. 205,454, filed June 10, 1988) to yield pAbT817.

pAbT817 is a vector for the insertion and expression of EHV-1 gB in vaccinia. pAbT817 contains the EHV-1 gB gene under the control of the vaccinia 7.5K promoter, the vaccinia TK gene for directing recombination in vaccinia, a lacZ gene under the control of the vaccinia BamF promoter for selection of recombinants and a bacterial replicon and ampicillin-resistance gene for growth and selection in E. coli.

EXAMPLE 4: Construction of monovalent IVR vector containing the EHV-1 gene encoding the glycoprotein gH homolog under the control of the vaccinia 7.5K promoter

The location of gene encoding the EHV-1 gH homolog was reported by Robertson et al., at the 12th International Herpesvirus Workshop. The information presented demonstrated that the coding sequence for the gene begins approximately 64 bp downstream from an EagI site located in the BamHI b fragment of the genome. In order to clone this gene, pAbT354, which contains the BamHI b fragment of EHV-1 cloned into the BamHI site of pEMBL18+, was digested with BamHI and the 15230 bp BamHI b fragment was gel-purified. This fragment was then digested with ClaI, and an approximately 5500 bp fragment was gel-purified. This DNA fragment was mapped with EagI and shown to contain two EagI sites. It was then partially digested with EagI, treated with Klenow, and ligated to a pEMBL18+ which had been digested with SmaI. The resulting plasmid was designated pAbT579.

pAbt579 was digested with KpnI and XbaI, and an approximately 3500 bp fragment was gel-purified. This fragment was ligated into the vector pAbt4532B, (an IVR vector described in U. S. Patent Application Serial No. 205,454, filed June 10, 1988, the teachings of which are incorporated by reference herein), which had been digested with KpnI and XbaI, to give the plasmid pAbT813.

pAbT813 is a vector for the insertion and expression of EHV-1 gH in vaccinia. pAbT813 contains the EHV-1 gH gene under the control of the vaccinia 7.5K promoter, the vaccinia TK gene for directing recombination in vaccinia, a lacZ gene under the control of the vaccinia BamF promoter for selection of recombinants and a bacterial replicon and ampicillin-resistance gene for growth and selection in E. coli.

EXAMPLE 5: Construction of an IVR vector containing the 40K vaccinia promoter for the insertion of foreign genes into the HindIII M region of vaccinia virus (Fig. 6)

The IVR vector pAbT4555 was described in U. S. Patent Application Serial No. 205,454, filed June 10, 1988. A derivative of this vector was constructed in the following manner. pAbT4555 was digested with HincII and BgIII, and a 110 bp fragment resulting from this digestion was gel-purified. In a second reaction, pAbT4555 was digested with SphI, treated with T4 DNA polymerase, and then digested with BgIII. A 3856 bp fragment resulting from this digestion was

gel-purified. The 110 bp fragment and the 3856 bp fragment were ligated to yield the plasmid pAbT4587.

pAbT4587 is a plasmid vector for use in IVR experiments in vaccinia. pAbT4587 is identical to pAbT4555, except that DNA sequences containing the 30K promoter that are present in pAbT4555 were deleted.

EXAMPLE 6: Construction of a monovalent IVR vector containing the EHV-1 gene encoding the glycoprotein gH homolog under the control of the vaccinia 40K promoter (Fig. 7)

pAbT813 was digested with BamHI and KpnI, and an approximately 3500bp fragment was gel-purified. This fragment was ligated to the IVR vector pAbT4587, which had also been digested with BamHI and KpnI, to generate pAbT829.

pAbT829 is a vector for the insertion and expression of EHV-1 gH in vaccinia. pAbT820 contains the EHV-1 gH gene under the control of the vaccinia 40K promoter, flanked by vaccinia DNA for directing recombination into the vaccinia HindIII M region, a portion of the vaccinia 29K host-range gene for selection of recombinants, and a bacterial replicon and ampicillin-resistance gene for growth and selection in E. coli.

EXAMPLE 7: Construction of a divalent IVR vector containing the EHV-1 gene encoding the glycoprotein gB under the control of the 40K promoter and the glycoprotein gH homolog under the control of the vaccinia 7.5K promoter (Fig. 8).

pAbT813 was digested with KpnI and treated with T4 DNA polymerase. SphI linkers (New England BioLabs cat. #1047) were ligated to the digested DNA, and the DNA was then digested with SphI. A 3765 bp fragment resulting from this digestion was gel-purified.

pAbT817 was digested with SphI, then ligated to the 3765 bp fragment from pAbT813. The resulting plasmid was designated pAbT822.

pAbT822 is a vector for the insertion and expression of EHV-1 gH and gB in vaccinia. pAbT822 contains the EHV-1 gH gene under the control of the vaccinia 7.5K promoter, the EHV-1 gB gene under the control of the 40K promoter, the vaccinia TK gene for directing recombination in vaccinia, a lacZ gene under the control of the vaccinia BamF promoter for selection of recombinants and a bacterial replicon and ampicillin-resistance gene for growth and selection in E. coli.

EXAMPLE 8: Construction of recombinant vaccinia viruses containing EHV-1 glycoprotein genes under the control of vaccinia promoters.

In vivo recombination is a method whereby recombinant vaccinia viruses are created (Nankano et al., (1982) Proc. Natl. Acad. Sci. USA 79:1593; Paoletti and Panicali, U.S. Patent NO. 4,603,112). These recombinant viruses are formed by transfecting DNA containing a gene of interest into cells which have been infected by vaccinia virus. A small percent of the progeny virus will contain the gene of interest integrated into a specific site on the vaccinia

genome. These recombinant viruses can express genes of foregin origin (Panicali and Paoletti. (1982) Proc. Natl. Acad. Sci. USA 79:4927; Panicali et al., (1983) Proc. Natl. Acad. Sci. USA 80:5364).

EHV-1 genes were inserted into the vaccinia virus genome at either the HindIII J or the HindIII M region, as listed in Table 1.

Vaccinia Recombinant	Vaccinia promoter/ EHV-1 gene(s)	Insertion site
vAbT184	7.5K/gH	TK
vAbT249	40K/gH	HindIII M
vAbT201	40K/gB	TK
vAbT243	40K/gB	TK
vAbT227	7.5K/gH + 40K/gB	TK
vAbT244	7.5K/gH + 40K/gB	TK

The TK gene is located in the HindIII J region. IVR vectors paBT813, pAbT817, or pAbT822 were transfected into Hul43TK$^-$ cells which have been infection with the NYCBH (TK+) strain of vaccinia virus (see Materials and Methods). The selection system for recombinant virus was bromodeozyuridine (BUdR), which is lethal for TK$^+$ virus but allows recombinant, TK$^-$ virus to grow. In addition, recombinant virus contains the lacZ gene which will metabolize Bluo-Gal and turn recombinant plaques blue. Therefore, blue, TK$^-$ plaques were picked and purified, and were shown, by Southern analysis, to contain the appropriate EHV-1 gene(s): vAbT184 contains gH; vAbT201 and vAbT243,

which are isolates from two separate IVRs, contain gB; and vAbT227 and vAbT244, which are isolates from two separate IVRs, contain gB and gH.

The 29K host-range gene is located in the HindIII M region (Gillard et al., (1986) Proc. Natl. Acad. Sci. USA 83: 5573) and has been developed as a selection system. Recombinant vaccinia virus vAbT33 contains the lacZ gene in place of the 29K gene. Therefore vAbT33 cannot grown on RK13 cells which require the 29K gene product, and, on permissive cells, is blue in the presence of BluoGal. See U.S. Patent Application Serial No. 205,189, filed June 10, 1988, which has been incorporated by reference herein.

IVR vector pAbT829 was transfected into BSC-40 cells which had been infected with vaccinia virus vAbT33 (See Materials and Methods). Recombinant viruses were selected as white plaques in the presence of BluoGal on RK13 cells. Plaques were picked and purified, and the recombinant designated pAbT249 shown, by Southern analysis, to contain the EHV-1 gH gene.

EXAMPLE 9: Black plaque assay for expression of EHV-1 antigens in recombinant vaccinia

The black plaque assay, described in Materials and Methods, is an in situ enzyme-based immunoassay which can detect protein expressed by vaccinia infected cells.

The black plaque assay was performed on vaccinia recombinants vAbT184, 201, 227, 243, 244, and 249,

using polyclonal rabbit anti-EHV-1 antiserum generated as described in Materials and Methods.

Plaques formed by the negative control, NYCBH virus, showed only a background color which was consistent with the background on the cell monolayer itself. Plaques formed by vaccinia recombinants vAbT201, 227, 243, 244, and 249 stained a distinct dark purple color which was much darker than the background on the cell monolayer, showing that these recombinants strongly express EHV-1 antigen(s). Black plaque assay of vAbT184 was weakly positive.

EXAMPLE 10: Immunoprecipitation of EHV-1 antigens from recombinant vaccinia (Fig. 9)

In order to examine the EHV-1 polypeptides synthesized by vAbT243 and vAbT249, immunoprecipitation analysis was performed as described in Materials and Methods. RK13 cells were infected with either vAbT243 or vAbT249 and labeled with either [^{35}S]-methionine or [^{3}H]-glucosamine.

In recombinant vAbT243, which contains the EHV-1 gB gene, a number of proteins were immunoprecipitated by polyclonal antiserum to total EHV-1. The pattern observed is very similar to that seen in a related herpesvirus pseudorabies virus (PrV), in which the glycoprotein homologous to HSV-1 gBm called gII in PrV, is present as a family of proteins, of apparent molecular mass 110, 68, and 55kD. These proteins represent a precursor form of the glycoprotein and the mature products derived from the cleavage of the precursor (Lukacs et al, 1985, J. Virol. 53:166;

Robbins et al., 1987, J. Virol., 61:2691). To examine the homology between the PrV family of gII glycoproteins and the glycoproteins encoded by the EHV-1 gB gene expressed in vAbT243, the immunoprecipation was also performed using monospecific goat anti-PrV gII antiserum obtained from L. Enquist, E.I. Dupont de Nemours & Co. The same pattern of protein expression was observed in these experiments, indicating that the protein synthesized in the recombinant is homologous to PrV gII.

Immunoprecipitation of [^{35}S]-methionine labeled cells infected with vAbT249 with polyclonal antiserum to total EHV-1 showed a single polypeptide of approximately 120kD. This molecular weight is similar to that of the HSV-1 gH glycoprotein, which is approximately 115kD. No protein was observed after immunoprecipitation of [^{3}H]-glucosamine labeled infected cells. However, it has been shown that some of the EHV-1 surface glycoproteins are not labeled during growth of EHV-1-infected cells in the presence of [^{3}H]-glucosamine (Turtinen and Allen, 1982, J. Gen. Virol. 63:481); the gH glycoprotein may be one of these.

EXAMPLE 11: Enzyme-linked immunosorbant assay (ELISA) with immune sera of mice immunized with recombinant vaccinia virus expressing EHV-1 antigens

Mice were immunized with vaccinia recombinants vAbT184, vAbT243, vAbT 244, or vAbT249, as described in Materials and Methods. Sera were obtained three and four weeks post-vaccination and tested against

vaccinia by ELISA, as described in Materials and Methods. Most mice exhibited a good anti-vaccinia immune response against the vaccinia recombinants (Table 2).

Anti-Vaccinia Titers of EHV-1/Vaccinia Sera

Virus	2 week bleed	3 week bleed	4 week bleed
vAbT 184	1:400	1:400	1:640
vAbT 243	1:2560	1:2560	1:2560
vAbT 244	1:1960	1:1920	1:2560
vAbT 249	1:960	1:2560	1:15360

EXAMPLE 12: Immunoprecipitations of EHV-1 antigens by immune sera of mice immunized with recombinant vaccinia virus expressing EHV-1 antigens (Fig. 10)

Immunoprecipitation analysis was performed on cells infected with EHV-1, using the mouse sera to vAbT243, vAbT244, and vAbT249 described in Example 11. Antisera to vAbT243 and vAbT244 precipitated a group of proteins, which, based on their migration in the polyacrylamide gel, appear to correspond to the EHV-1 gB family of proteins described in Example 10. Antisera to vAbT244 and vAbT249 failed to precipitate a protein corresponding to EHV-1 gH; however, it is not known whether this is because these recombinants failed to elicit an antibody response to the gH glycoprotein, or because gH is minor component of

EHV-1 virions and so is not detected in this assay. The results of the plaque reduction assay, described in Example 13, favor the latter explanation.

EXAMPLE 13: EHV-1 neutralization assay with immune sera of mice immunized with recombinant vaccinia virus expressing EHV-1 antigens (Fig. 11)

Antisera to vAbT243, vAbT244, and vAbT249, described in Example 11, were assayed for their ability to neutralize EHV-1 virus infectivity in vitro, as described in Materials and Methods. All three antisera inhibited plaque formation by EHV-1 on RK13 cells, while control normal mouse serus did not, demonstrating that antisera raised against recombinant vaccinia viruses that express EHV-1 glycoproteins gB and/or gH neutralize EHV-1.

Plasmid deposits

The plasmids pAbT243, pAbT244 and pAbT249 have been placed on depisit at the American Type Culture Collection (ATCC) and have been assigned the following ATCC accession numbers:

PLASMID	ATCC #
pAbT817	40479
pAbT822	40478
pabT829	40477

Equivalents

Those skilled in the art will recognize or be able to ascertain, using no more than routine experimetation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed within the scope of this invention.

Claims

1. A recombinant pox virus capable of expressing in a host one or more immunogenic proteins of equine herpesvirus-1.

2. A recombinant pox virus of Claim 1, which is of the species vaccinia.

3. A recombinant pox virus of Claim 1, wherein the immunogenic protein is an envelope glycoprotein of equine herpesvirus-1.

4. A recombinant pox virus of Claim 3, wherein the envelope glycoprotein of equine herpesvirus-1 is the gB or gH glycoprotein or immunogenic subunits or fragments thereof.

5. A recombinant vaccinia virus containing, in a region of the viral genome nonessential for replication of the virus, one or more DNA sequences encoding the protein components of envelope glycoproteins of equine herpesvirus-1, the DNA sequence or sequences being under control of individual vaccinia promoters.

6. A recombinant vaccinia virus of Claim 5, wherein the envelope glycoprotein is the gB or gH glycoprotein, or immunogenic subunits or fragments thereof.

7. A DNA vector for insertion of EHV-1 DNA sequences and for subsequent in vivo recombination with pox virus to produce a recombinant pox virus capable of expressing the EHV-1 DNA in a host, comprising:

a. one or more pox virus promoter linked to;

b. DNA sequences encoding an EHV-1 antigens, each DNA sequence being under control of a different pox promoter; and;

c. DNA sequences of the pox virus flanking the construct of elements a and b, the DNA sequences being substantially homologous to a region of the pox viral genome which is nonessential for replication of the pox virus;

d. a replicon for replication in a prokaryotic host; and

e. a structural gene encoding a marker or indicator for selection of prokaryotic hosts transformed with the vector, the gene being under control of a prokaryotic promoter.

8. A vector of Claim 7, wherein the DNA sequences encoding an EHV-1 antigen encode the gB, gH or the gB and gH antigen.

9. A vector of Claim 8 for recombination with vaccinia virus, wherein the pox virus promoters are the 11K promoter, the 7.5K promoter, the 40K promoter, the BamF promoter or the 30K promoter of vaccinia virus and where the flanking DNA sequences are sequences homologous to the thymidine kinase gene of vaccinia virus.

10. A vector of Claim 7, further comprising:
f. a pox virus promoter linked to a structural gene which encodes a marker or indicator for selection of recombinant pox virus, the promoter and structural gene being located between the flanking pox viral sequences.

11. A vector of Claim 10, for recombination with vaccinia virus, wherein the pox virus promoter is the vaccinia BamF promoter and the structural gene encoding a marker or indicator is the E. coli lacZ gene encoding beta-galactosidase.

12. A vector of Claim 11, wherein the DNA sequences encoding the EHV-1 antigen encode the gB, gH or gB and gH antigen.

13. The DNA vector pAbT813.

14. The DNA vector pAbT817.

15. The DNA vector pAbT822.

16. A vector for insertion of EHV-1 DNA sequences and for subsequent in vivo recombination with pox virus to produce a recombinant pox virus capable of expressing the polypeptides encoded by the EHV-1 DNA in a host, comprising:

a. one or more pox virus promoters linked to:

b. DNA sequences encoding EHV-1 antigens, each DNA sequence being under the control of a different pox virus promoter;

c. DNA sequences of the pox virus flanking the construct of elements a and b, the DNA sequences being substantially homologous to a region of the pox viral genome which is nonessential for replication of the pox virus.

d. a replicon for replication in a prokaryotic host; and

e. a structural gene encoding a marker or indicator for selection of prokaryotic hosts transformed by the vector, the gene being under control of a prokaryotic promoter.

17. A vector of Claim 16, wherein the pox virus promoter is selected from the group consisting of the 7.5K promoter, the 40K promoter, the BamF promoter, the 11K promoter and the 30K promoter.

18. A vector of Claim 16, for recombination with a pox virus that contains a mutation in the 29K gene, wherein the flanking DNA sequences are:
a. homologous to the Hind IIIM region of vaccinia virus; and
b. capable of restoring the function of the 29k gene in the recombinant viruses.

19. A vector of Claim 16, for recombination with vaccinia virus, wherein the pox virus promoter is the vaccinia 40K promoter and the EHV-1 DNA sequences are those encoding the gH glycoprotein.

20. The DNA vector pAbT829.

21. A method of vaccinating a host animal against equine herpesvirus-1, comprising inoculating the animal with recombinant pox viruses capable of expressing an immunogenic protein or proteins of equine herpesvirus-1.

22. A method of Claim 21, wherein the recombinant pox virus is a vaccinia virus capable of expressing one or more envelope glycoproteins of equine herpesvirus-1.

23. A method of Claim 22, wherein the glycoproteins are gB or gH of equine herpesvirus-1.

24. The method of Claim 21, wherein the recombinant pox virus is vAbT243, vAbT244, vAbT249, or mixtures thereof.

25. A method of producing antigenic proteins of equine herpesvirus-1, comprising the steps of:
a. infecting cells with a recombinant pox virus capable of expressing an antigenic protein of equine herpesvirus-1;
b. culturing the cells under conditions which allow the virus to replicate and to express the antigenic protein;
c. isolating the antigenic protein from the cells.

26. A method of Claim 25, for producing the gH or gB glycoproteins of equine herpesvirus-1, wherein the cells are infected with a recombinant vaccinia virus capable of expressing gH or gB proteins of equine herpes virus-1 or immunogenic fragments or subunits thereof.

27. A method of producing antibody against antigenic proteins of equine herpesvirus-1, comprising the steps of:
a. inoculating an animal with a recombinant pox virus capable of expressing an antigenic protein of equine herpesvirus-1.
b. isolating serum containing antibody raised against the antigenic protein.

28. A method of producing monoclonal antibody against antigenic proteins of equine herpes virus-1, comprising the steps of:

a. immunizing an animal with a recombinant pox virus capable of expressing an antigenic protein of equine herpesvirus-1;

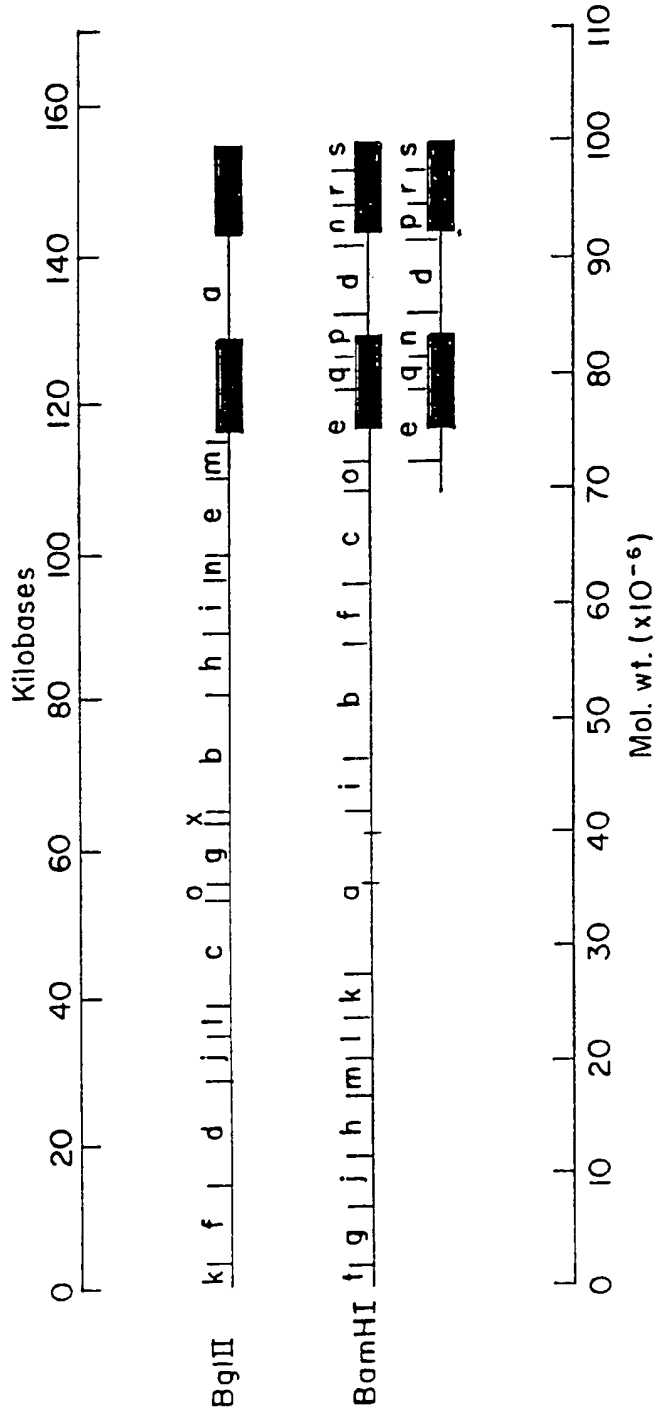
b. obtaining antibody-producing cells from the animal;

c. fusing the cells with an immortalizing cell to produce fused cell hybrids;

d. selecting fused cell hybrids which produce antibody against the antigenic protein; and

e. growing the selected fused cell hybrids and obtaining antibody secreted by the hybrids.

29. Recombinant vaccinia viruses, vAbT243, vAbT244 and vAbT249.


Kilobases
0
20
40
60
80
100
120
140
160
BglII
k f d j l c o g x b h i n e m a
BamHI
t g j h m l k a i b f c o e q p d n r s
e q n d p r s
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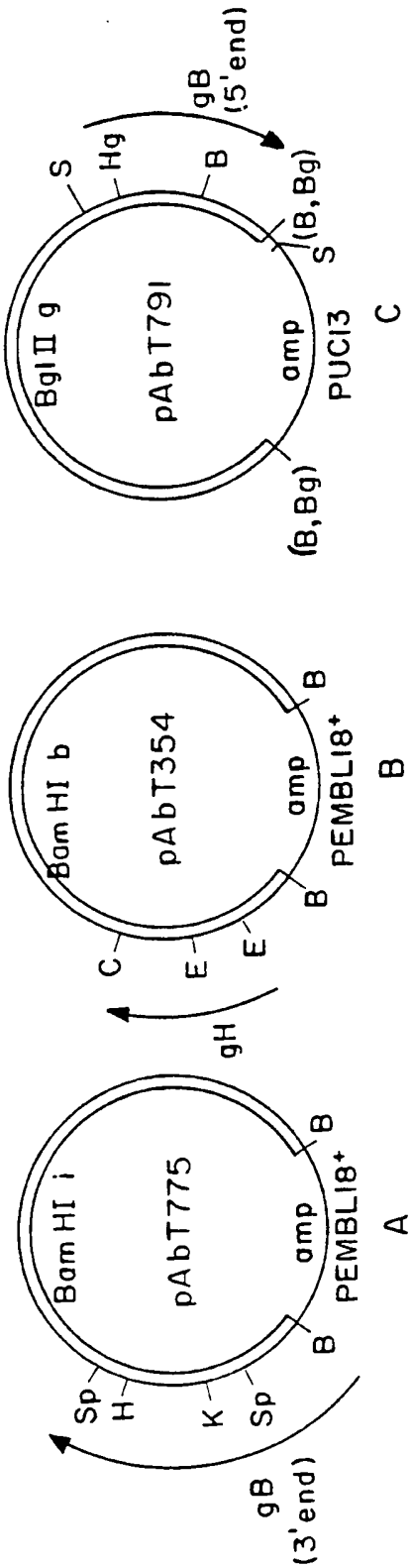
20
30
40
50
60
70
80
90
100
110
Mol. wt. (x10⁻⁶)

FIG. 1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S
Hg
B
(B,Bg)
S
BglII g
pAbT791
amp
PUC13
(B,Bg)
C
BamHI b
pAbT354
amp
PEMBL18+
B
B
C
E
E
gH
BamHI i
pAbT775
amp
PEMBL18+
B
B
Sp
H
K
Sp
gB (3'end)
A
B: BamHI
Bg: BglII
C: ClaI
H: HindIII
Hg: HgaI
K: KpnI
S: SacI
Sp: SspI

FIG. 2

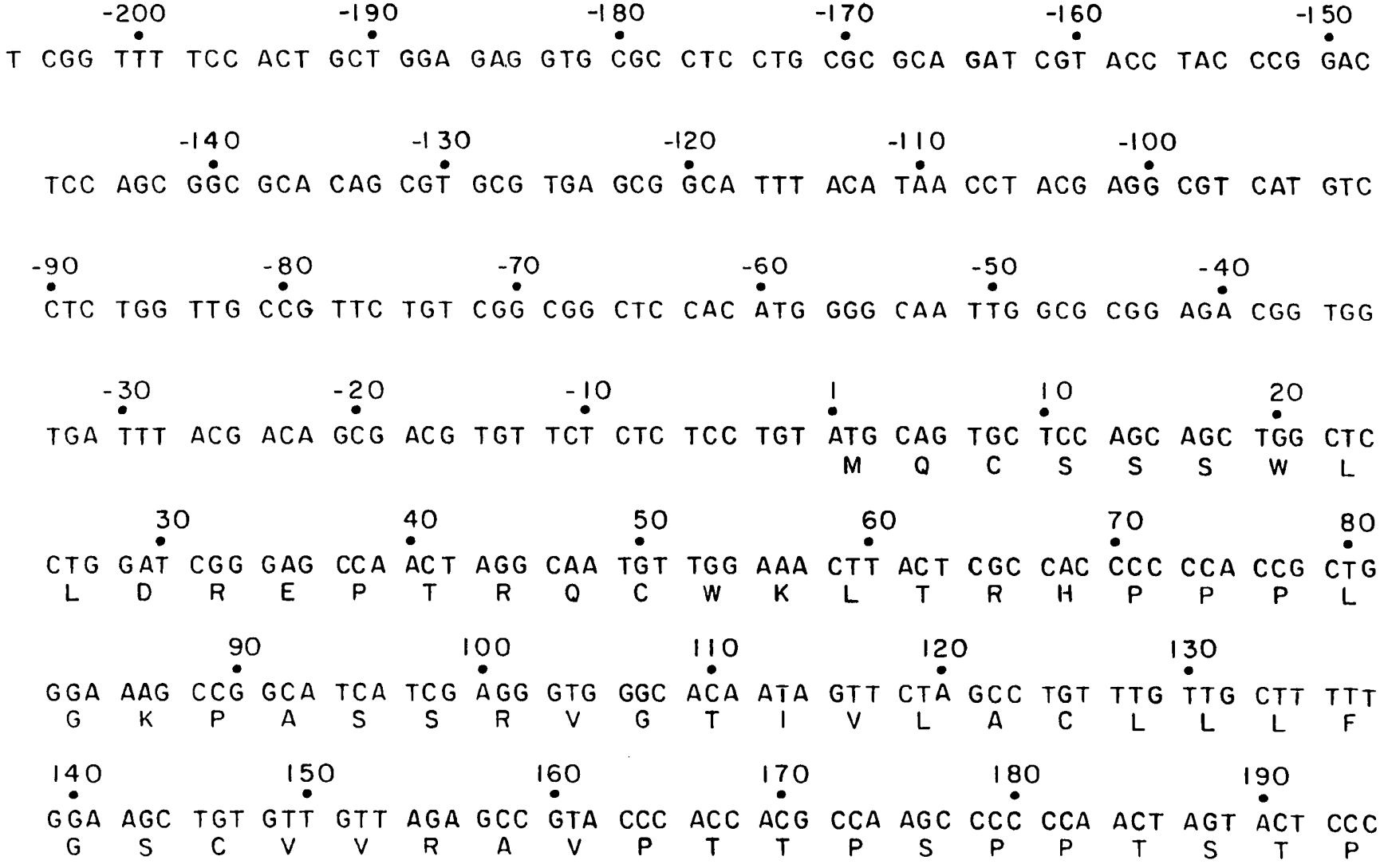


FIG. 3 (Sheet 1 of 6)

SUBSTITUTE SHEET

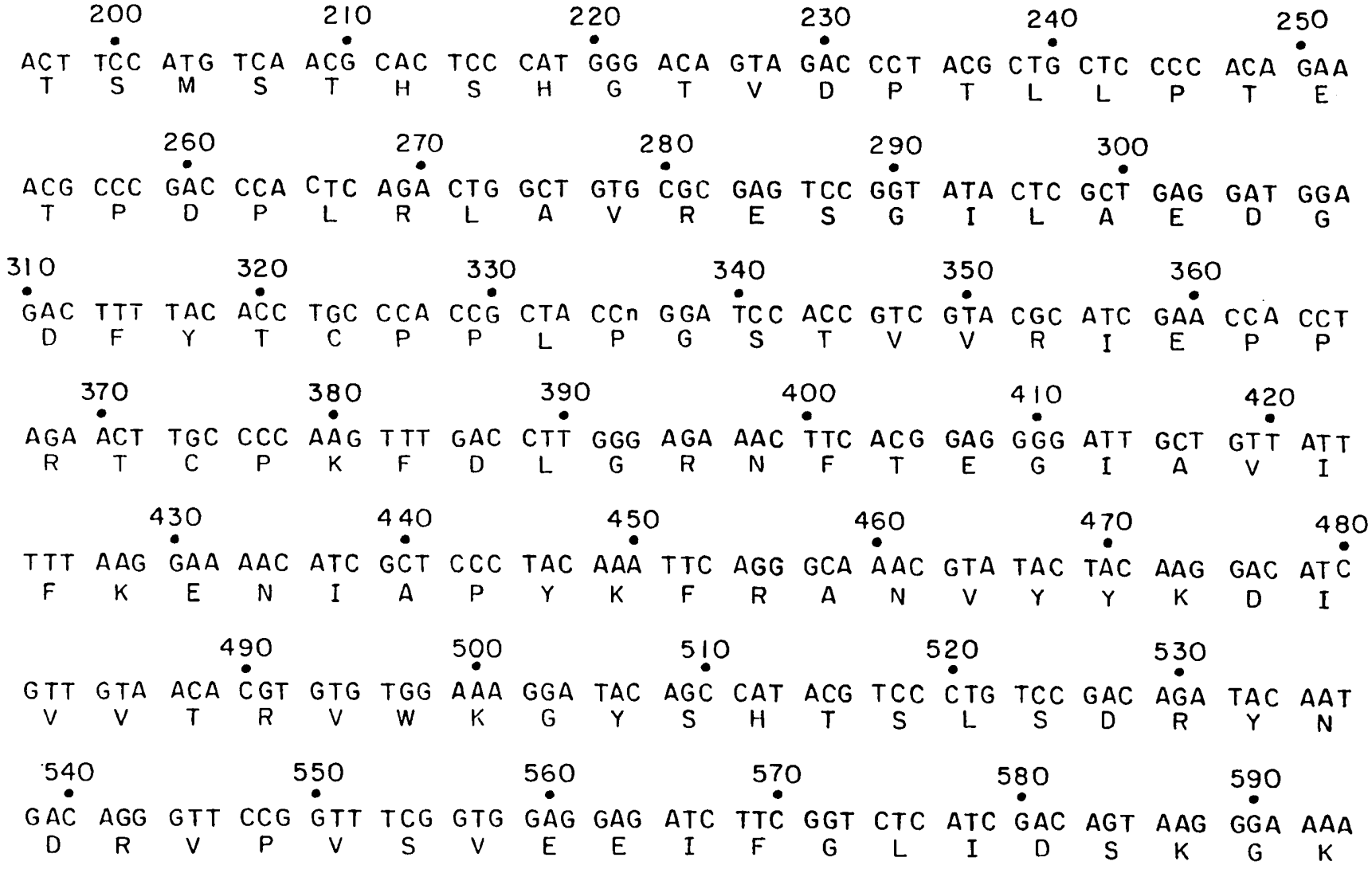


FIG. 3 (Sheet 2 of 6)

SUBSTITUTE SHEET

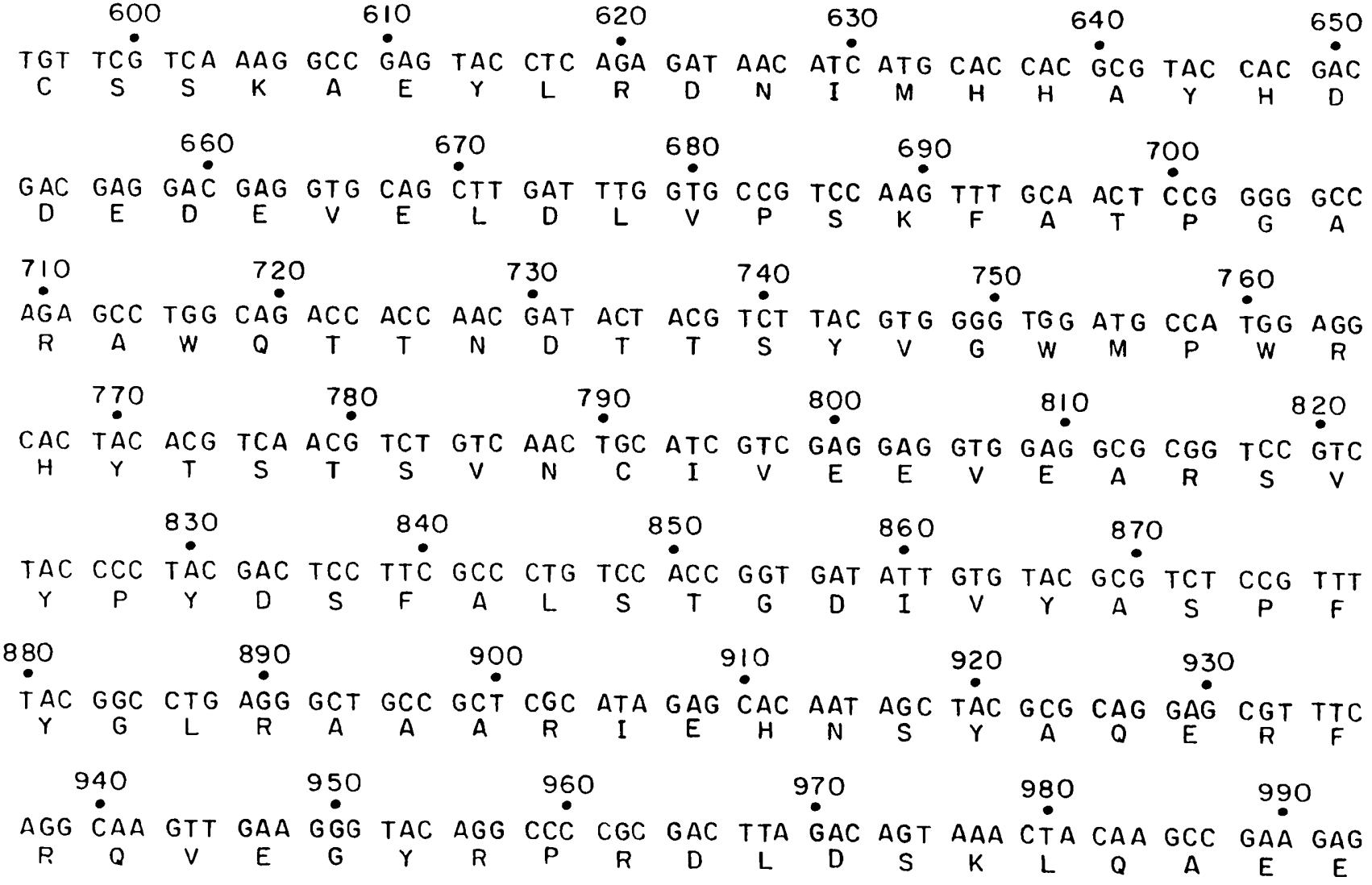


FIG. 3 (Sheet 3 of 6)

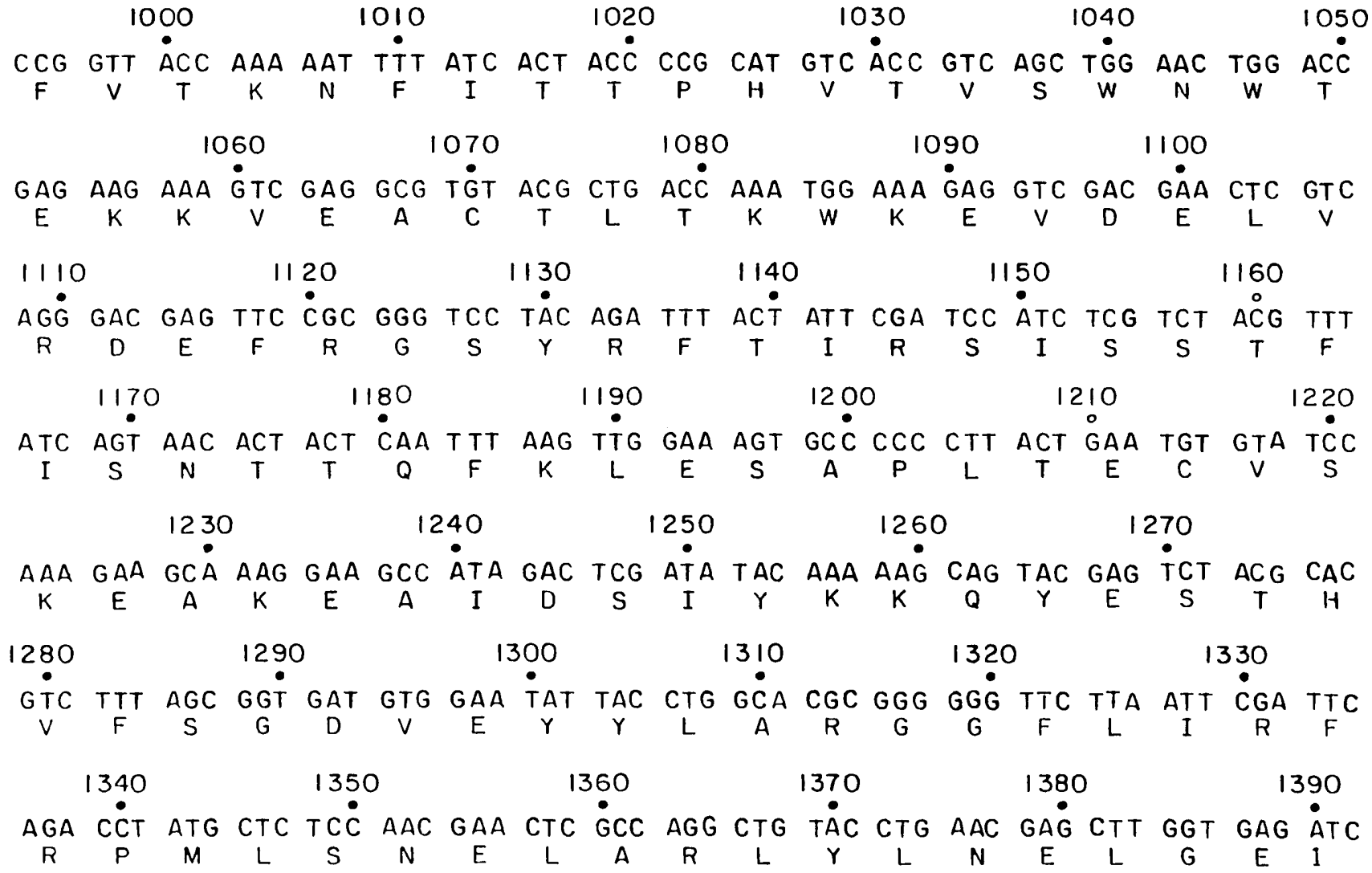


FIG. 3 (Sheet 4 of 6)

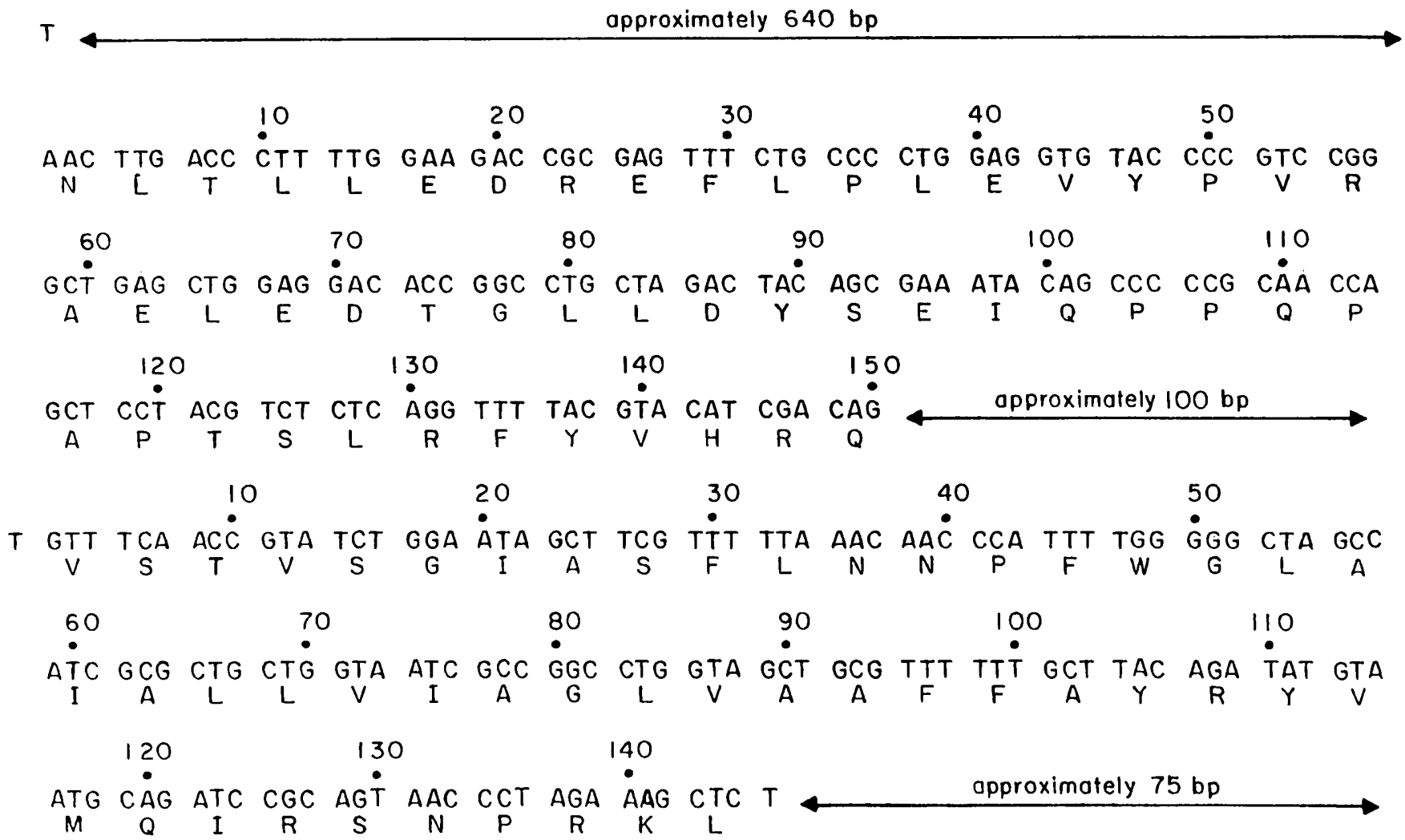


FIG. 3 (Sheet 5 of 6)

SUBSTITUTE SHEET

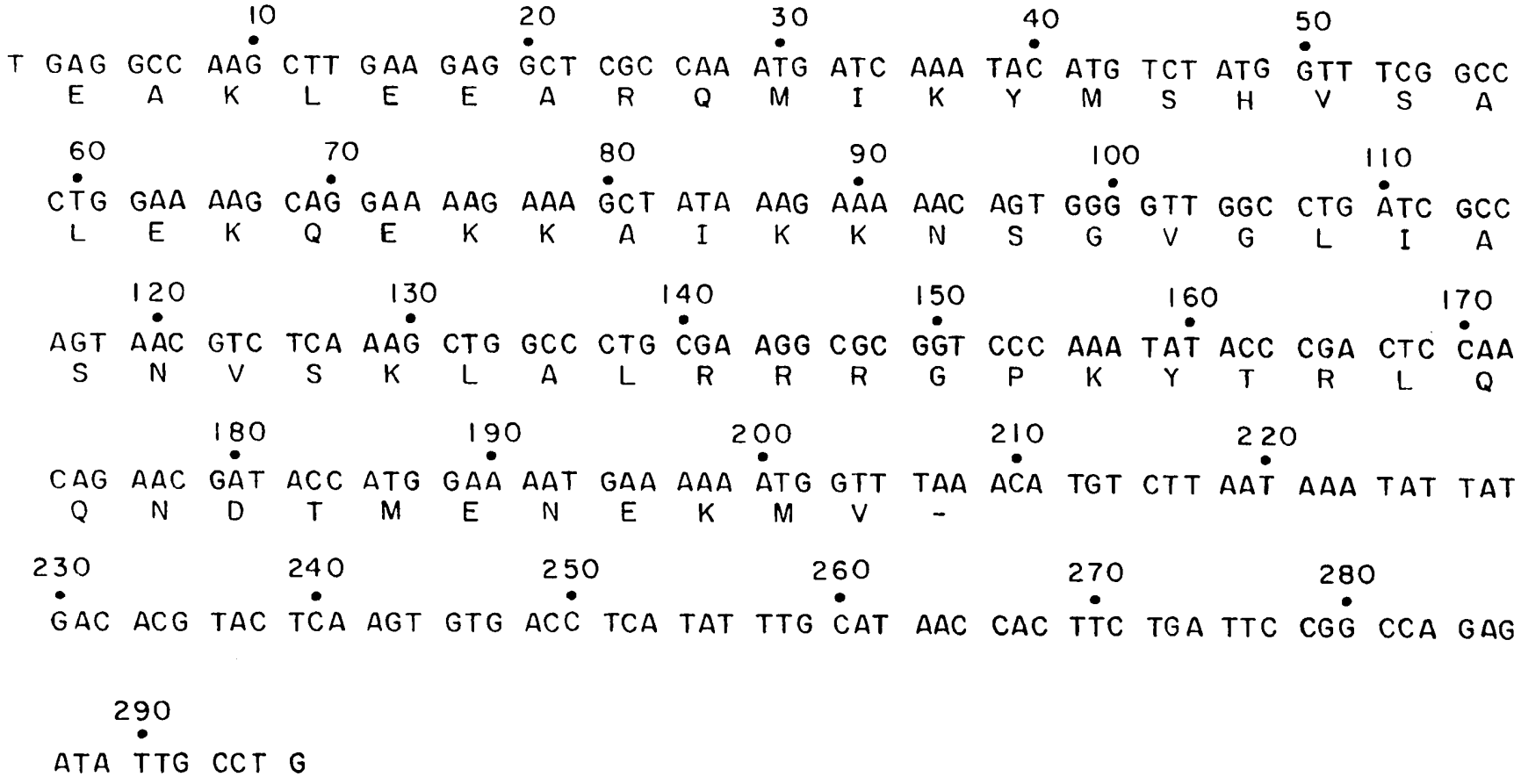


FIG. 3 (Sheet 6 of 6)

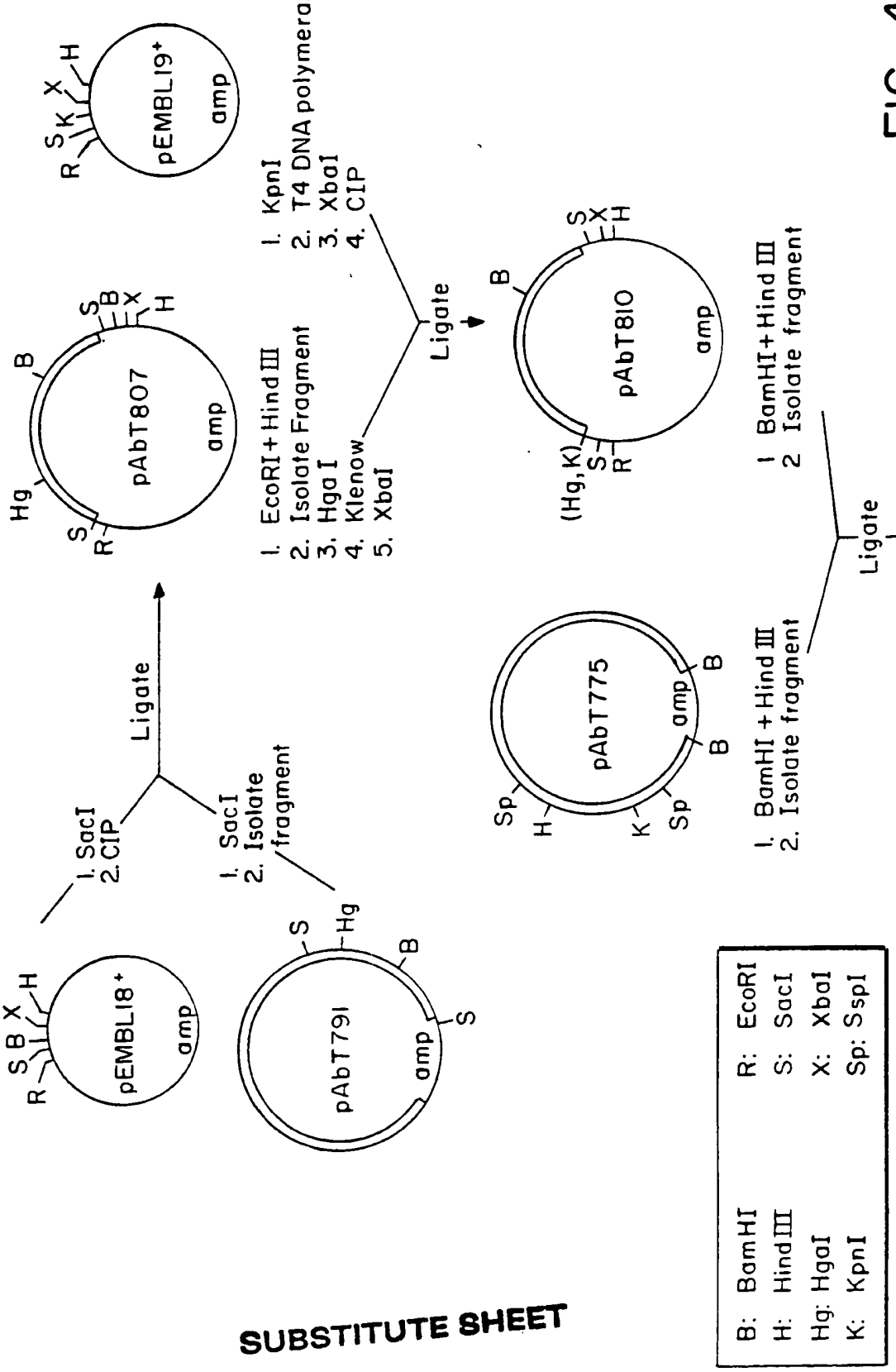


FIG. 4

SUBSTITUTE SHEET

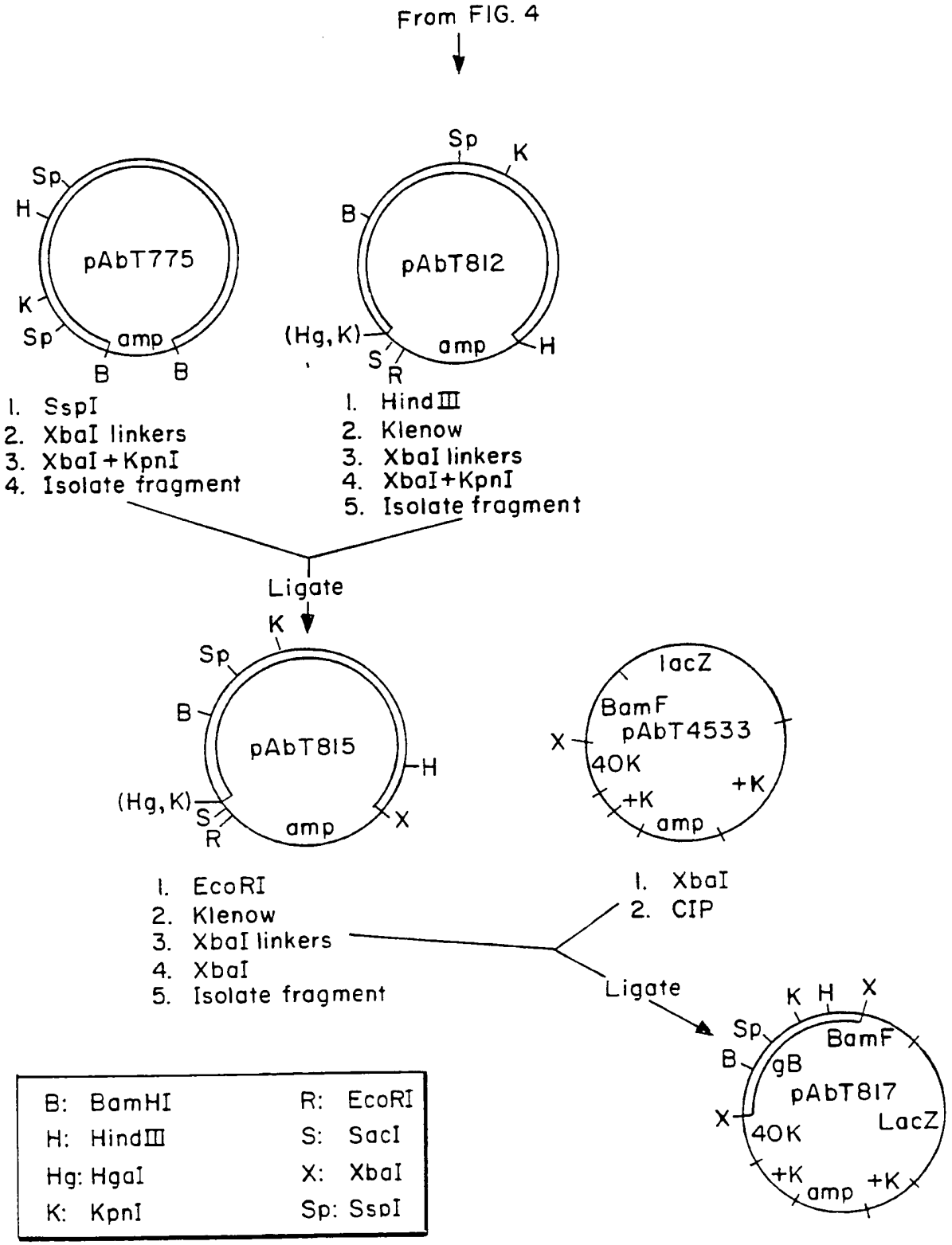
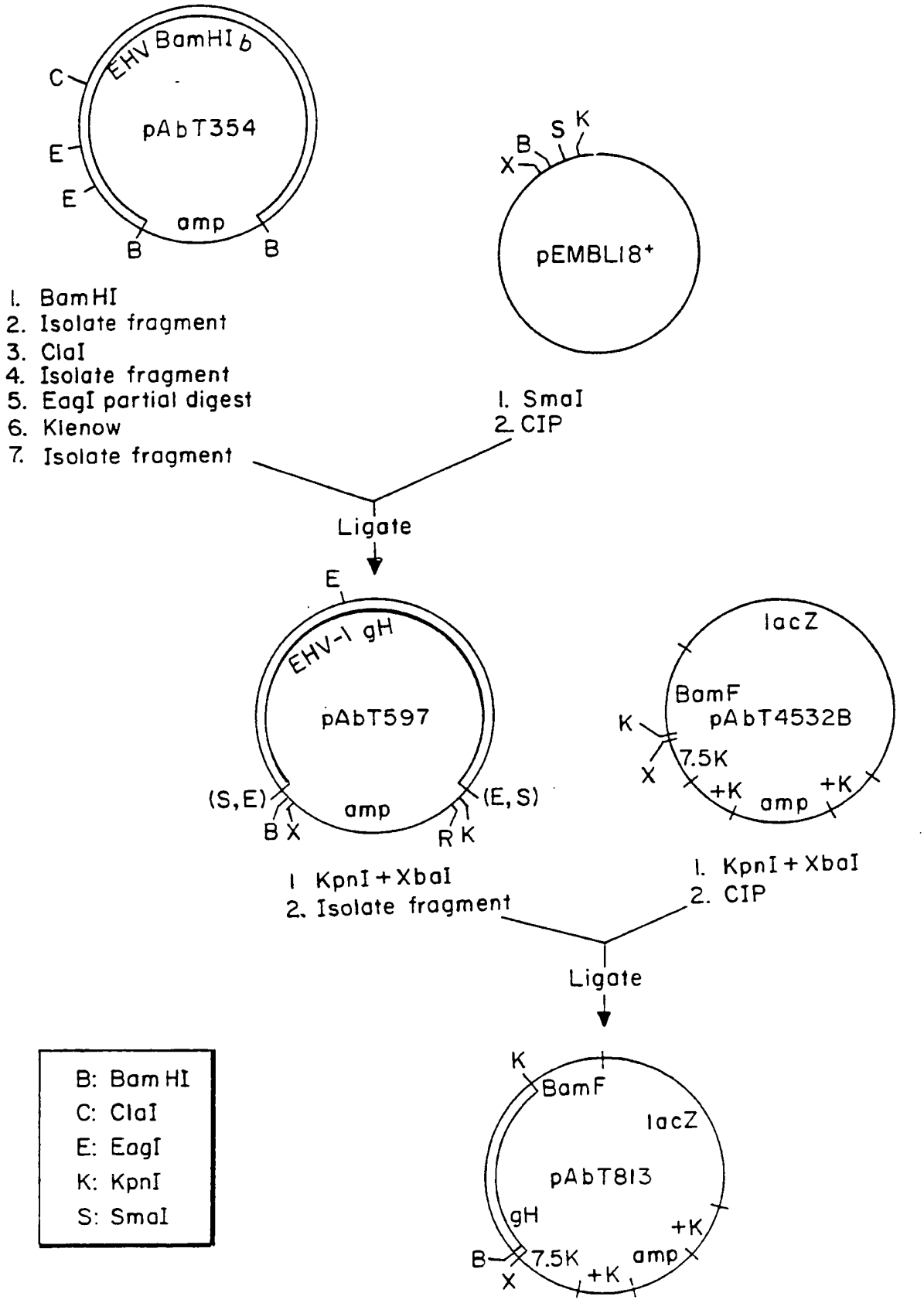


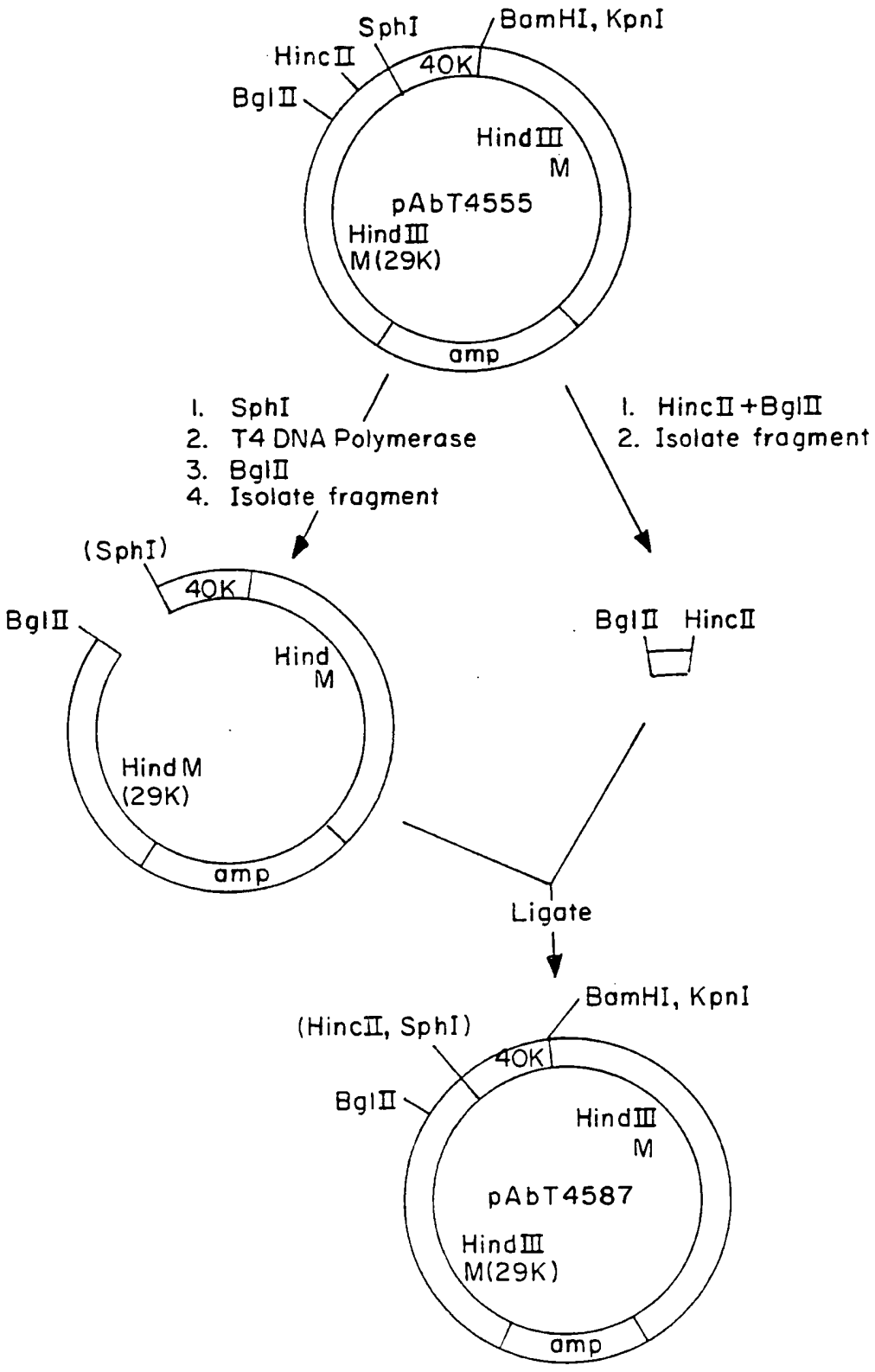
FIG. 4 (Cont)

SUBSTITUTE SHEET



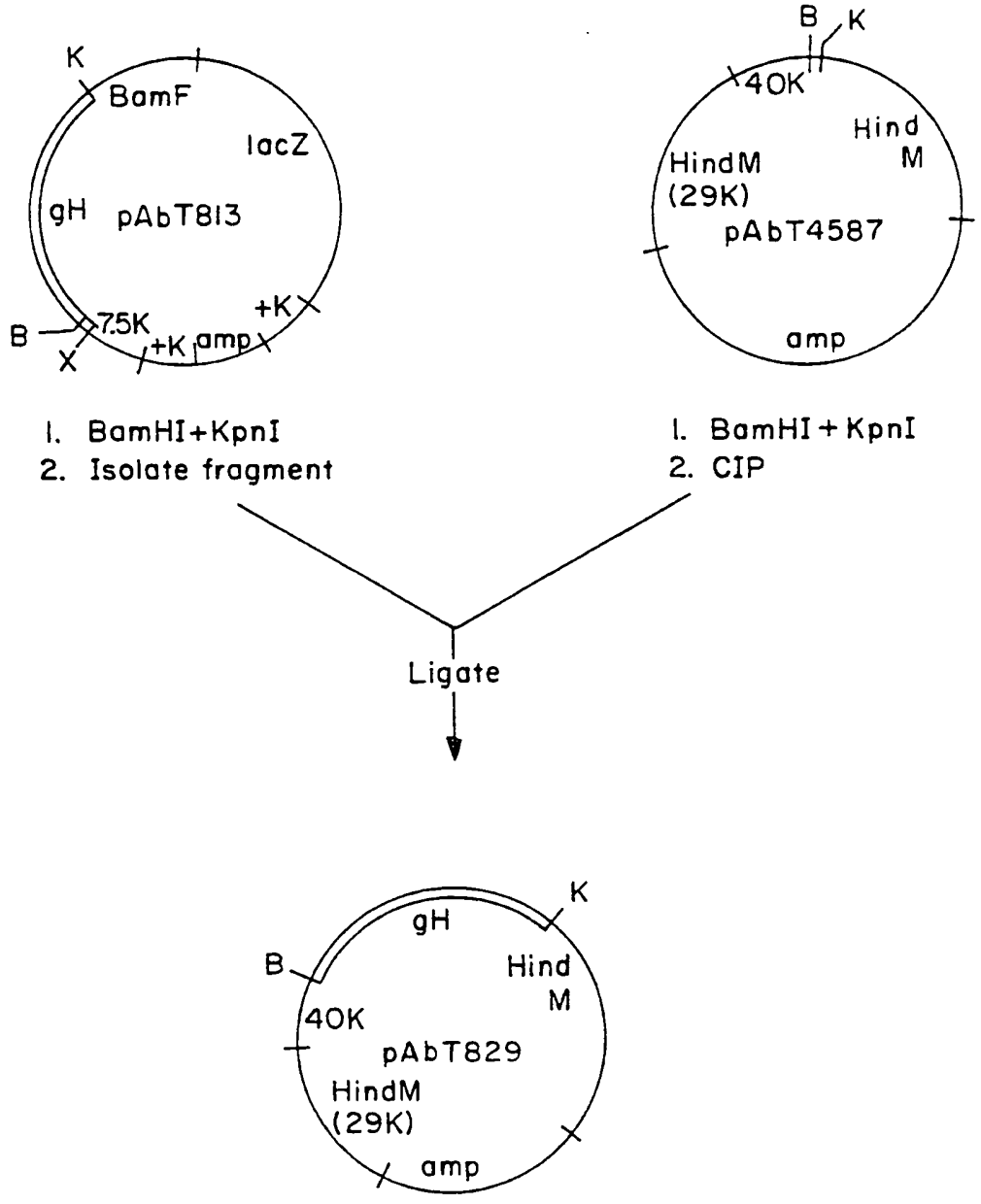
SUBSTITUTE SHEET

FIG. 5



SUBSTITUTE SHEET

FIG. 6



B: BamHI
K: KpnI
X: XbaI

FIG. 7

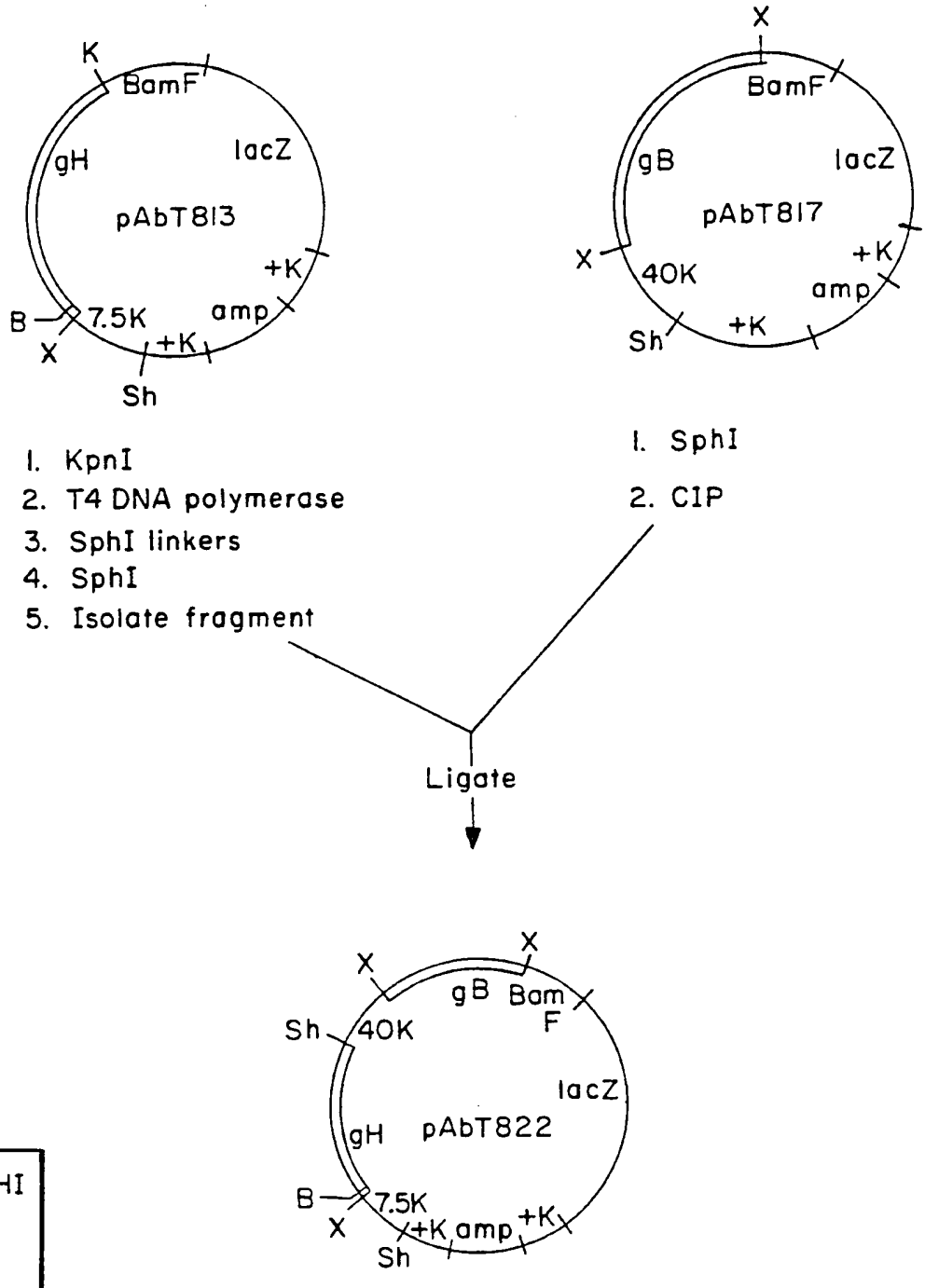

K
BamF
gH
lacZ
pAbT813
+K
B
7.5K
amp
X
+K
Sh
X
BamF
gB
lacZ
pAbT817
X
40K
+K
amp
Sh
+K
1. KpnI
2. T4 DNA polymerase
3. SphI linkers
4. SphI
5. Isolate fragment
1. SphI
2. CIP
Ligate
X
X
gB
Bam
F
Sh
40K
lacZ
gH
pAbT822
B
7.5K
X
+K
amp
+K
Sh
B: BamHI
K: KpnI
Sh: SphI
X: XbaI

FIG. 8

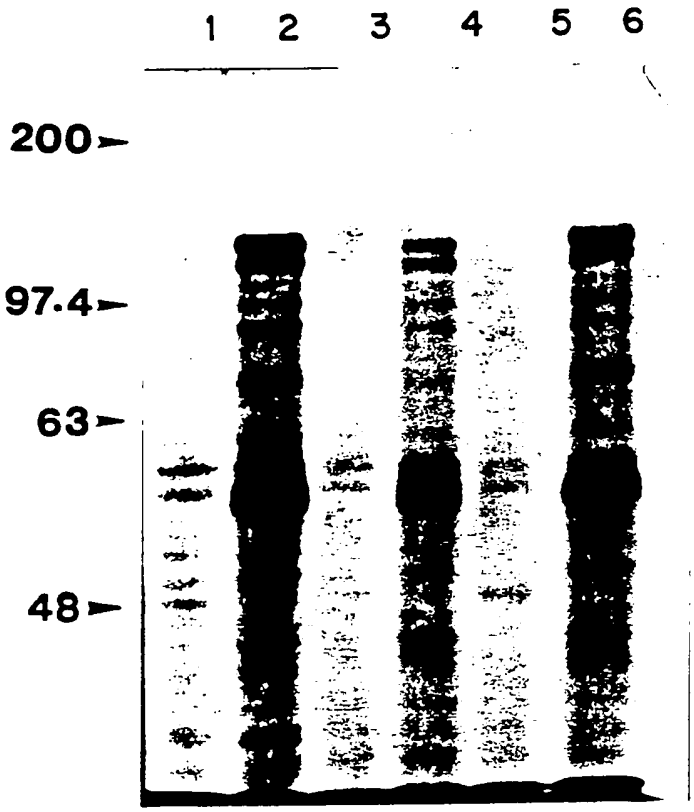


FIG.9A

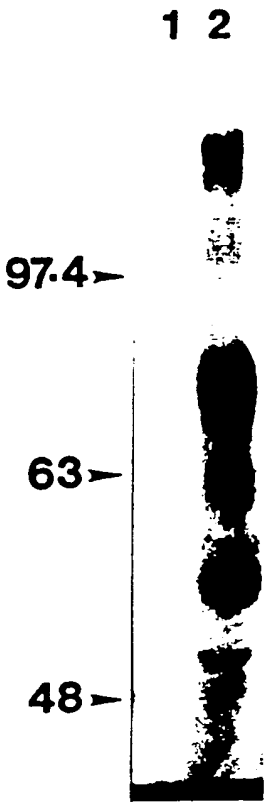


FIG.9B

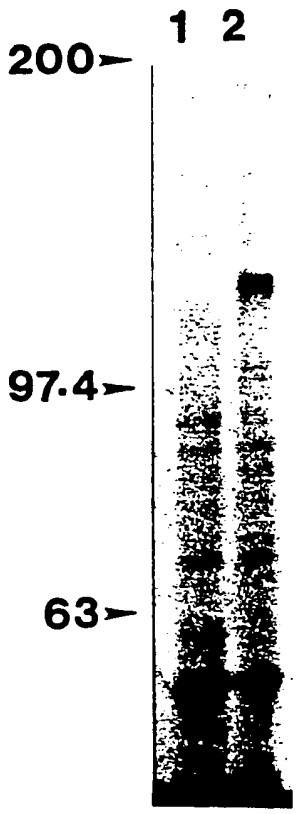


FIG.9C

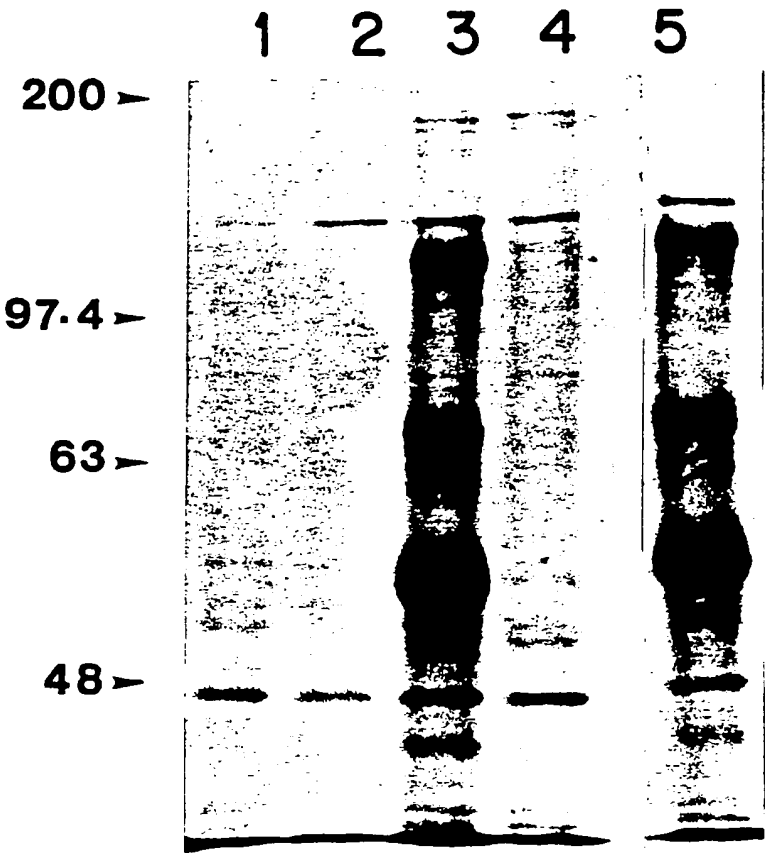


FIG.10

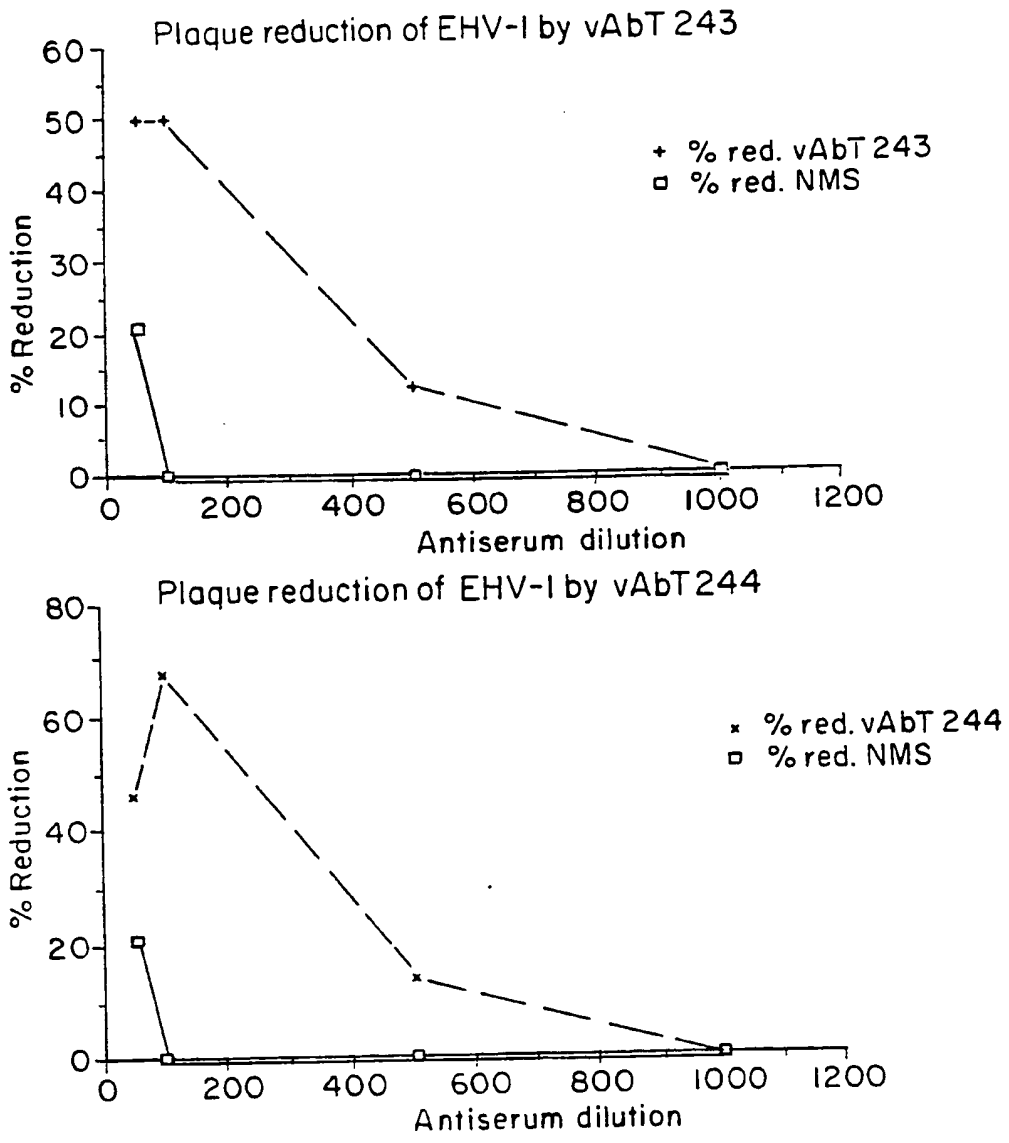

Plaque reduction of EHV-I by vAbT 243
% Reduction
Antiserum dilution
+ % red. vAbT 243
□ % red. NMS
Plaque reduction of EHV-I by vAbT244
% Reduction
Antiserum dilution
× % red. vAbT 244
□ % red. NMS

FIG. 11

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/03362

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC [5] C 12 N 15/00, A 61 K 39/245, A 61 K 39/285, A 61 K 39/295, C 12 P 21/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC [5]	C 12 N, A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Journal of Cellular Biochemstry, UCLA Symposia on Molecular & Cellular Biology, 30 January - 26 February 1988, Abstracts of the 17th Annual Meetings, Supplement 12B, 1988, Alan R. Liss, Inc. (New York, US), J.M. Whalley et al.: "The gene in equid herpesvirus 1 analogous to the herpes simplex virus gene encoding a major envelope antigen glycoprotein gB", page 24, abstract no. F 215, see the abstract	1-6,25,26
Y	EP, A, 0261940 (APPLIED BIOTECHNOLOGY INC.) 30 March 1988, see the whole document cited in the application	1-19,25,26, 29
Y	Journal of Virology, vol. 61, no. 8, August 1987, American Society for Microbiology, G.P. Allen et al.: "Use of lambdagt11 and monoclonal antibodies to map the ./.	1-19,25,26, 29

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27th October 1989	04 DEC. 1989
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	F.M. VRIJDAG

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

	genes for the six major glycoproteins of equine herpesvirus 1", pages 2454-2461, see the whole article cited in the application --	
P,X	J. Gen. Virol, vol. 70, 1989, SGM (GB), J.M. Whalley et al.: "Identification and nucleotide sequence of a gene in equine herpesvirus 1 analogous to the herpes simplex virus gene encoding the major envelope glycoprotein gB", pages 383-394, see the whole article ----	1-6,25,26

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers * ..... because they relate to subject matter not required to be searched by this Authority, namely:

* 20-24,27,28 See PCT-Rule 39.1(IV); Method of treatment of the human or animal body by therapy, as well as diagnostic methods.

2. [ ] Claim numbers ....., because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers ....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this International application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the International application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 8903362 SA 30442

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 24/11/89
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0261940	30-03-88	JP-A- 63245670	12-10-88